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**Effects of Voluntary Exercise on Prefrontal-Dependent
Set-Shifting in Adolescent Rats**

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Abstract

Physical exercise has been shown to have positive impacts on executive functioning (working memory, inhibitory control, cognitive flexibility) in humans of all ages. Rodent studies provide further insight into how exercise may impact the extent of the cognitive benefits for adult versus adolescent rodents. Preliminary data from our laboratory suggest that exercise may improve cognitive flexibility in adolescent rats. In the current experiment, we directly compare exercising versus non-exercising adolescent and young adult rats in cognitive flexibility. We hypothesized that adolescent rats with access to a home cage running wheel would perform best in a maze-based cognitive flexibility task. We also examined whether or not these hypothesized benefits remain after exercise stops. We did not find support for our hypothesis; exercise had no detectable effect on cognitive flexibility in adolescent or young adult rats. We discuss some reasons why we might not have observed exercise-related effects in the current study, such as low running distances seen in the exercising rats when compared to prior studies.

Introduction

Physical exercise has a multitude of positive impacts on the cognitive abilities of humans. The effects of exercise can enhance cognition across multiple age groups, ranging from school age children to the elderly (Vos et al. 2013). Children ranging from seven to nine years of age (resembling early adolescence) have been of particular interest in the context of exercise and cognition. Cross-sectional studies, as well as randomized controlled studies, have demonstrated that physical activity is positively related to executive control, with more active children exhibiting superior attention, decision-making ability, and differential brain function compared with their less active peers (Pontifex et al., 2011; Chaddock-Heyman et al., 2013; Hillman et al., 2014).

Participating in physical activity during childhood can enhance the function of prefrontal cortex areas important for goal-oriented cognitive control (Pontifex et al., 2011; Hillman et al., 2014). In a nine-month study of children aged eight-nine years old, children were divided into either a physical activity intervention group or a wait-list control group. The children in the exercising group participated in an after school program consisting of one hour of exercise (moderate to vigorous aerobic activity with some muscle and bone strengthening exercises), five days per week. The control group was not contacted after group assignment but they were told they would have the opportunity to join the exercise group the next year. Both intervention and control groups were tested on a cognitive control task both before and at the termination of the exercise period. During this cognitive control task, children had five shapes presented to them and were instructed to look at the middle shape and to respond as quickly and accurately as they could to the changing location of arrows pointing to shapes. If the arrow pointed to the left, participants would press a button with their left index finger, whereas if the arrow pointed right,

participants would press the button with their right index finger. A specific condition, the incongruent flanker trial condition, required attentional and interference control to filter potentially misleading flankers that were associated with incorrect behavioral responses. Exercising children had decreases in fMRI activation in the right anterior prefrontal cortex during a cognitive control task, whereas children who did not participate in the same exercise program had unchanged activation results (Chaddock-Heyman et al, 2013). Less brain activation may reflect more mature brain function; studies show decreased activation and superior performance on cognitive tasks in adults compared to children (Booth et al., 2003). Thus, it can be concluded that exercising during childhood may promote maturation of the prefrontal cortex, which plays a role in goal-oriented cognitive control.

In a similar study by Hillman et al. (2014), children aged seven to nine years old were divided into two groups: a nine-month afterschool physical activity program or a wait-list control in which children did not participate in any exercise. Attentional inhibition and cognitive flexibility tasks were used to collect information on response accuracy and reaction time. A modified flanker task was used to assess attentional inhibition; during this task children were given a variety of fish-shaped objects and were asked to press a button as quickly as they could based on the direction of the fish in relation to the fish in the middle. Cognitive flexibility was measured using a color-shape switch task, during which children were presented with characters of different shapes and colors and were asked to make a judgment on either the color or shape of the character. They were then asked to switch their decision in regards to shape and color, which requires the greatest amount of executive control. The results showed that in the attentional inhibition task, the exercising group had greater improvement from pretest to post test compared to the non-exercising group, although response accuracy increased in both groups. In the

cognitive flexibility tasks, the exercising group had better response accuracy when they were asked to switch their decision from their initial judgment of shape or color, indicating that those children were demonstrating greater executive control. The study ultimately suggests that daily physical exercise recommendations caused increased attentional inhibition as well as cognitive flexibility among children.

Rodent studies have been conducted to mimic human models to explore the relationship between environmental enrichment manipulations, age, and cognitive flexibility, as well as their neurobiological underpinnings. For example, exposure to an enriched environment during adolescence, but not during adulthood, has been shown to improve spatial learning and memory (Lores-Arnaiz et al., 2007). As a result of these manipulations, there is likely morphological and functional reorganization that occurs during adolescence (including myelogenesis, axonogenesis, and maturation of brain regional neurocircuitry), specifically in regions of the brain involved in learning and memory, the hippocampus and the prefrontal cortex (PFC) (Counotte et al., 2010). For example, exercise has the ability to produce neurobiological changes in the medial PFC (mPFC) such as shortened apical dendrite branch length and greater spine density, during adolescence (30-44 days old during exercise) compared to adulthood (Eddy & Green, 2017). In addition, exercise in adolescents can produce longer lasting cognitive effects compared to adults, such as retained object recognition two and four weeks after termination of exercise (Hopkins, Nitecki, & Bucci, 2011). Neurobiological changes, including changes in dendrites and astrocytes, that occur during adolescence as a result of exercise lead us to believe that adolescent rats with access to voluntary exercise may show more cognitive benefits than adult rats.

Few studies have directly compared adolescent and adult rodents in regards to the effect of voluntary exercise. The age range for adolescent rats is generally considered to be between

approximately 28 and 42 days old (Spear, 2000). This is the age range that rats would be expected to exhibit adolescent-typical characteristics such as undergoing growth spurts. Rats are considered young adults by approximately 50 days of age (Spear, 2000). To the author's knowledge, there are only three studies that directly compared the behavioral and neurobiological effects of exercise on adult and adolescent rodents (Hopkins et al. 2011; Jin et al., 2017; Eddy & Green, 2017). All three of these studies have generally concluded that exercise has more profound effects on the cognition of adolescent rodents compared to adults. In one of these studies, adolescent rats exercised between the ages of 32-60 days old and adult rats exercised between the ages of 63-91 days old. Both adult and adolescent rats were given access to running wheels for voluntary exercise for 4 weeks and were then tested for their object recognition as well as for brain derived neurotrophic factor related (BDNF; related to neural plasticity) levels in the perirhinal cortex and hippocampus (Hopkins et al. 2011). During the object recognition task, rodents were placed into a box with an object for 10 minutes and then the next day were placed with the same object, as well as with a new object, into the same box. The amount of time the rodent spent with the novel versus old object was recorded. It was found that both exercising adolescent and adult rats had improved object recognition, with a higher mean discrimination ratio between time spent with novel and familiar items when compared to non exercising rats. Exercising adolescent and adult rats also had increased BDNF levels in the hippocampus and perirhinal cortex immediately following 4 weeks of exercise. For adult exercising rats, improvements in object recognition were not seen 2 weeks after the rats no longer had access to the running wheels. Adolescent rats, however, were still able to differentiate between novel and familiar objects 2-4 weeks after they no longer had access to voluntary exercise. This suggests that some cognitive benefits of exercise are only transiently seen in adult

rodents when compared to adolescent rodents and that adolescent rats have longer lasting cognitive benefits as a result of exercise.

Another comparison between the effects of exercise in adolescent versus adult rats showed that adolescent rats have improved spatial learning ability via increased hippocampal neurogenesis after 6 weeks of daily exercise (Jin et al., 2017). Upon arrival to the colony, the adolescent rats were approximately 35 days old and the adult rats were approximately 140 days old. At the time of testing after 6 weeks of running, adolescent rats were approximately 77 days old and the adult rats were approximately 182 days old. Spatial learning ability was tested using a radial 8-arm maze apparatus where rats were deprived of water for 24 hours and were then allowed to explore the maze to seek water at the end of the arms. This test was finished once rats found all of the water rewards or after 5 minutes elapsed. It was found that the non-exercising adolescent rats made more errors, such as reentering previously visited arms, than adult rats in the radial maze task, but exercising young adult rats made fewer errors. It was also found that there was more active hippocampal neurogenesis in exercising rats compared to non-exercising rats. Most importantly, exercising adolescent rats were found to have more active neurogenesis compared to exercising adult rats. Hippocampal neurogenesis is associated with improvement of learning ability and memory function (Kim et al., 2014), and so it can be hypothesized that increased neurogenesis in exercising adolescent rats can cause improvement in spatial learning ability.

There is evidence that voluntary exercise, especially during adolescence, can improve cognitive flexibility, which has medial prefrontal cortex underpinnings (Eddy & Green, 2017). The prefrontal cortex has been found to undergo neural alterations during adolescence not only in humans, but also in species ranging from rodents to non-human primates. The prefrontal

cortex is largely involved in higher level cognitive functioning, including cognitive flexibility, and is one of the last parts of the brain to be fully developed (Spear, 2000). However, the density of spines and pyramidal cells declines between adolescence and adulthood (Mrzljak et al., 1990). Pyramidal cells comprise about two-thirds of all excitatory neurons in the cerebral cortex of mammals (Spruston, 2008), and dendritic spines receive most of the excitatory inputs that enter the pyramidal cells. This loss of spines can be seen as a “sculpting” process, in which unnecessary neural connections present during adolescence decrease into adulthood as connections and processing become more efficient.

Cognitive effects of voluntary exercise with not only adolescent but also adult rodents have been linked to prefrontal cortex function. Prefrontal cortex functioning in rats, mice, nonhuman primates, and humans can be measured using cognitive flexibility tasks. Eddy and Green (2017) studied the effects of exercise on ABA renewal, with testing occurring 2 weeks after adult and adolescent rodents were given access to locked or unlocked running wheels. Adolescent rats were 21 days old on arrival to the colony and 30 days old at the time of testing; adult rats were 47 days old on arrival to the colony and 56 days old at the time of testing. Rats were given lever press acquisition in Context A (with lever presses rewarded with a sucrose pellet), extinction in Context B, and were then tested in both Context A and Context B. Although responding was extinguished in Context B, rats “renew” responding in Context A. ABA renewal of extinguished instrumental behavior has medial prefrontal cortex (mPFC) underpinnings (Trask, Shipman, Green, & Bouton, 2017). This is confirmed by lesioning the mPFC and then testing the rats on the same task to see reduced performance. It was found that exercising adolescent rats had reduced ABA renewal compared to non-exercising adolescent rats but exercising adults did not differ from non-exercising adults. In addition, it was shown that

exercising during adolescence, but not adulthood, was associated with a greater apical dendrite spine density of pyramidal neurons in the mPFC (Eddy & Green, 2017). This provides evidence that there are morphological changes in the mPFC occurring in adolescent rats secondary to exercise. Ultimately, this study suggests that exercise may have an important influence on the medial prefrontal cortex structure and function during adolescence in rats.

Attentional set-shifting tasks can also be used to measure cognitive flexibility in the context of voluntary exercise. An experiment by Brockett et al. (2015) provided adult male rats with or without ad lib access to running wheels for 12 days as a means of voluntary exercise. The attentional set-shifting task tested rats in their ability to discriminate between different digging media and/or textures covering digging containers to retrieve a food reward. This task consisted of several different discriminations, specifically, simple discrimination (SD), compound discrimination (CD), intradimensional shift (IDS), reversal (REV), and extradimensional shift (EDS) involving the mPFC. An extradimensional shift occurs when a rat learns to be rewarded for one dimension and then is rewarded on a different stimulus dimension while having to ignore the previously rewarded stimulus. The EDS in this case involved changing from media to texture covering the digging container between testing days. Each rat needed to reach a total of 6 correct trials consecutively in order to proceed to the next phase. The results of this experiment were that running seemed to enhance cognitive flexibility, given that rats with access to running wheels required fewer trials to reach a criterion of 6 consecutive correct trials and committed fewer errors in an extra dimensional shift.

Set-shifting and cognitive flexibility can also be measured in humans. An example of a prefrontal dependent task is the Wisconsin Card Sort Task (WCST) in humans, which requires subjects to sort cards according to periodically shifting rules based on stimulus categories that

include color of symbols, shape of symbols, and number of shapes. The classification rule changes every 10 cards and so this task is ultimately measuring how well people can adapt to the changing rules. Participants with schizophrenia and ADHD, for example, demonstrate increased perseverative behavior during the task. They are able to learn the first sorting rule just as quickly as control subjects, although they have difficulty suppressing responding to the initial rule once the category has shifted and the first sorting rule is no longer correct (Goldberg et al. 1987).

Prefrontal cortex functioning and set shifting abilities can also be measured in rodents using a T maze-based extra-dimensional set-shifting task (Eddy et al. 2013; Stefani et al. 2003). The T maze-based extra-dimensional set-shifting task is a cognitive flexibility task, which requires rats initially to learn one rewarded stimulus–response strategy, and then to change their response strategy according to a different set of stimulus parameters. This T-maze configuration is created when the arm across from the start arm that the rat is placed in is blocked off. Each arm is a different combination of two dimensions, brightness and texture. Thus, the arms are: black/smooth, black/rough, white/smooth, and white/rough. Stefani et al. (2003) as well as Eddy et al. (2013) implemented use of this maze task to measure prefrontal function of rodents (see Figure 1). Once the rat is habituated to the maze, the set-shifting procedure begins, which comprises two daily sessions, Set 1 and Set 2. On the first day of testing, rats are trained on Set 1, which consists of either a brightness or texture discrimination task, with the other stimulus dimension being irrelevant. Rats are trained to a criterion level of 8 correct consecutive trials on Set 1 to avoid overlearning of the first stimulus, which could potentially affect rat performance of Set 2. The next day the rats are trained on Set 2, which is the stimulus dimension that the rats were trained to ignore in Set 1. During Set 2 testing, the stimulus from Set 1 is potentially distracting. Rats undergo Set 2 for 80 trials to allow for enough trials to compare the learning

curves between the groups. Stefani et al. (2003) showed that Set 2 requires mPFC. Prefrontal function is thus measured by the ability of the rodent to successfully adapt their response to a correct stimulus dimension after being rewarded for a different stimulus dimension during testing the day prior.

Eddy et al., (2013) used this T-maze task to study the effects of voluntary exercise on the ability of both adult male and female rats to perform a set-shift task. These rats were between the ages of 59-63 when they arrived in the colony and were between the ages of 65-79 when exercising. Rats assigned to the exercise group had access to voluntary exercise via a running wheel and then underwent testing after 2 weeks of exercising. The results showed that exercise only positively affected the performance of the male and female rats in Set 1, with the exercising rats reaching 8 correct trials in a row in fewer trials than the non-exercising rats. There was no difference seen in trials to criterion between exercising and non-exercising rats in Set 2. This result suggests that exercise in young adults might not have effects on mPFC function, similar to the results of Eddy and Green (2017).

The foundation for the current experiment was provided by Eddy et al. (unpublished). This study explored the impact of voluntary exercise in adolescent and young adult rats on discrimination learning and set-shifting in the T-maze extra-dimensional set-shifting task. This study consisted of two experiments, experiment 1 being two weeks of voluntary exercise when rats were 30-44 days old and experiment 2 being voluntary exercise when rats were 44-58 days. Different rats were used for each of these experiments. The results showed that exercising adolescent rats showed facilitated set-shifting compared to non-exercising adolescent rats while exercise had minimal effects on set-shifting in young adult rats. There was no difference between groups in discrimination learning (Set 1) in either experiment, with no significant difference

between each of the groups in the number of trials to reach 8 correct consecutive trials. This suggested that voluntary exercise during adolescence might have effects on the mPFC compared to a lack of effects of voluntary exercise on mPFC during adulthood. However, a major limitation was that adolescent and young adult rats were tested in separate experiments conducted at different times. In addition, the experiments simultaneously compared the effects of exercise versus methylphenidate on set-shifting performance, and so all rats underwent daily injections of either methylphenidate or vehicle; this also adds complexity to the interpretation of the results.

In the current experiment, we used a 2x2 factorial design with young adult and adolescent rats, either with access to an unlocked running wheel or a locked running wheel, to directly compare the effects of exercise on adolescent versus adult rats. Previous studies have shown that physical activity in adolescent rats is associated with dendritic and astrocytic changes in the mPFC when compared to adults, as well as voluntary exercise improving performance on cognitive flexibility tasks in adolescent rats compared to adults (Eddy & Green, 2017). We are ultimately interested in whether adolescent exercising rats have an increased set shifting ability when compared to adolescent non-exercising rats, as well as longer lasting cognitive effects of the exercise, in comparison to adults. We hypothesize that adolescent rats with access to a home cage running wheel will perform best in the T maze-based extra-dimensional set-shifting task, with fewer trials to criterion (i.e. number of trials to reach 8 consecutively correct choices) in Set 2. We also hypothesize that the adolescent, exercising rats will have better retention even after a period of no exercise. Given some of our previous results, it can be inferred that adult exercising rats will perform better than non-exercising adult rats in Set 1 (Eddy et al., 2013). However, the rats in Eddy et al. (2013) exercised when they were 65-79 days old but previous research from

Eddy et al. (unpublished) indicated that there was no effect of exercise when rats were 44-58 days old. It is thus unclear what the effects of exercise for the young adult rats in the current experiment may be.

Methods

Subjects. A total of 48 male Wistar rats obtained from Charles River Canada were used, aged approximately 22-25 days old (adolescent) or 36-39 days old (adult) when arriving in the colony. They were tested at approximately the ages 44 and 58 days old, respectively. All rats of the same age were initially housed in groups of two for 5 days to acclimate to the colony. All animals were kept in the same colony room, which was maintained at a controlled temperature and humidity with a 12/12-hour light/dark cycle. After 6 days, rats were individually housed in cages based on whether they had access to voluntary exercise via a running wheel, which was either locked or unlocked. This creates the following groups for the experiment (1) Adolescent, Exercise; (2) Adolescent, No Exercise; (3) Adult, Exercise; (4) Adult, No Exercise. From arrival to the colony, the rats had 7 days of *ad lib* access to food and water. On the seventh day, baseline weights were obtained and rats began being food deprived to 90% of their free feeding weight in preparation for training in the T-maze task. Target weights for adolescent rats were determined by taking 90% of their projected normal weight based on age in the growth curve for the male Wistar rat. All procedures involving rats were approved by the University of Vermont institutional animal care and use committee (Protocol 11-036).

Voluntary exercise. Rats assigned to the exercise group were given unlocked running wheels in their home cage and non-exercise rats were given identical wheels that were locked in place to control for environmental enrichment effects. Adolescent rats were approximately 27-30 days old upon beginning exercise and between 41-44 days old at the culmination of the 14-day

exercise period. Adult rats exercised between the ages of 41-44 until 55-58 days old. Each running wheel was 36 cm in diameter. The running wheels were identical and produced by Med Associates Inc. (St. Albans, VT). Once placed in their home cages, rats had 24 hour *ad lib* access to running wheels for two weeks. Each running wheel had an automatic counter attached to it that recorded each revolution of the wheel. Running wheel counts were measured once every 24 hours, at the same time each day. These wheel count measurements, originally measuring wheel rotations, were converted into kilometers run for each rat in the exercise group.

T-maze apparatus. The T-maze was placed on a table in a small, quiet room with minimal overhead lighting. The maze was constructed of painted polycarbonate, and consisted of a square central platform (each side measured 14.0 cm), to which four arms were attached. Each arm was 14.0 cm wide, 40.6 cm long, and 20.3 cm high. A food well was located 2.5 cm from the end of each arm, measuring 1.9 cm in diameter and 0.63 cm deep. This was constructed in a way that the rat could not visually detect the food pellets from the arm entrance. Each arm was a different combination of two dimensions, brightness and texture. Two of the arms were painted black, while two of the arms were painted white. Two of the arms had a smooth texture, while the other arms had a rough texture. The rough texture was created by mixing a small amount of sand into the paint. This ultimately created the following combinations of arms: black/smooth, black/rough, white/smooth, and white/rough. The central platform was painted grey. A polycarbonate insert, which was also grey, was positioned between the central platform and any one of the arm entrances. The T-maze configuration was created when the arm across from the start arm that the rat was placed in was blocked off. Lastly, a gray polycarbonate holding chamber (35.6 cm x 35.6 cm x 35.6 cm) was used to place rats into during the inter-trial intervals (ITIs).

Habituation. After 5 days of acclimation to the colony, rats were handled daily. During the first 2 days of the handling regimen, rats were handled for approximately 5 minutes and then weighed. Food restriction began on the third day, with handling of rats limited to lifting them from their cages to the scale. Each rat was weighed and then fed between 2-4 food pellets (or more if necessary) in order to maintain their weight at 90% of their target weight. For adolescent rats, this changed daily based on the rat growth curve. On the seventh day of food restriction, rats reached their target weights, and habituation training began. Prior to the first day of habituation, there was 1 g of 45-mg sucrose pellets (Purina TestDiet) placed into each rat cage to acquaint them with the pellets. These sucrose pellets served as activity reinforcements. The first phase consisted of “open-arm” habituation, which was designed to familiarize the rats with the maze and pellets. On the first day of open-arm habituation, 16 pellets total were placed inside the maze, specifically 4 pellets in the food well of each arm. Each rat was allotted 10 minutes to freely explore the maze and eat the pellets. At the culmination of the 10 minutes, the rat was placed into a holding chamber for two minutes. During that time, the remaining pellets were counted and then disposed of. After two minutes, the rat was returned to the holding chamber and both the holding chamber and the maze were cleaned with an odor elimination solution (Nok-Out). On the second day of open-arm habituation, there was 1 pellet placed into each of the four food wells. Rats were allotted 5 minutes to explore the maze and eat the pellets, but were removed from the maze if they ate all the pellets in less than 5 minutes. Rats were again placed in the holding chamber for two minutes afterwards. On the third day of open maze habituation, there was again 1 pellet placed into each of the four food wells. Rats were allotted 2 minutes to explore the maze and eat pellets, or until they had consumed all the pellets. They were placed in the holding chamber for 2 minutes at the conclusion of the session. All rats proceeded to the next

phase of habituation regardless of whether they ate all the pellets at the culmination of the third open-arm habituation.

The next phase was “blocked arm habituation”, which consisted of two consecutive days and eight trials each day. Time to complete the 8-habituation trials was recorded on both days. The goal of this phase was to familiarize the rat with the T configuration of the maze, as well as with being moved quickly between the maze and the holding chamber. To create the “T” shape, a polycarbonate insert was placed between the central platform and one of the arms. There was a predetermined start arm number for each of the eight trials in a way that each of the start arms, numbered 1-4, was used twice in a random order. The maze was rotated so that the start arm changed for each trial, with different left and right arm choices for trial as well. One of the arms (left or right) was baited with a pellet in the food well. Rats were placed at the food well of the start arm (which was not baited) and then ran up the start arm. Once they got to the center of the maze they had the option of turning left or right to pick an arm. If they correctly chose the baited arm then they would eat the pellet, but if they went down the un-baited arm then they were given a few seconds to inspect the empty food well before being taken out of the maze. When rats were removed from the maze between trials, they were placed into the holding chamber for approximately 15 seconds. While the rat was in the holding chamber, the maze was rotated to switch the start arm, the polycarbonate insert was placed across the new start arm, and a new pellet was placed in the rewarded arm. In the second trial, the new rewarded arm where the pellet was placed was opposite the arm where the pellet was in the first trial. The rat was again placed in the start arm, allowed to choose one of the arms, obtain a reward if the correct arm was chosen, and was removed to the holding chamber for about 15 seconds. This was repeated for another 6 trials for a total of 8 trials. For the remainder of the trials, the arms were baited

randomly so that there was an equal probability of being rewarded for any arm choice. It was not the goal of this phase for the rats to make associations between arm choices and rewards, so if the rats obtained the pellet reward for three consecutive trials, a pellet was not placed in the maze for the next trials so that neither arm was baited. This same procedure was performed a second day.

Set-Shift Procedure. Once the rat was habituated to the maze, the set-shifting procedure to measure cognitive flexibility comprised two daily sessions, Set 1 and Set 2. At the start of Set 1 testing, the adolescent rats were 40-43 days old, and the adult rats were 54-57 days old. Prior to the testing, rats were randomly assigned to a stimulus dimension they were rewarded for. For rats paying attention to the texture dimension, half were rewarded for choosing smooth textured arms and the other half were rewarded for choosing rough textured arms. For rats paying attention to the color dimension, half were rewarded for choosing white colored arms and the other half were rewarded for choosing black colored arms. Before the start of Set 1, each rat was placed into the holding chamber for 2 minutes. Afterwards, the rat was placed in the start arm and was allowed to make an arm choice once in the center of the maze. If the rat correctly chose the rewarded stimulus, they would obtain the pellet in the food well of that arm. If the rat chose incorrectly, the rat would not find a pellet in the food well and was removed from the maze into the holding chamber for 15 seconds. During that time the maze was rotated and re-baited. This same procedure was repeated until the rat reached a criterion of 8 correct consecutive choices.

Set 2 was conducted the day after Set 1. In Set 2, the rats must pay attention to the opposite dimension than in Set 1. For example, a rat rewarded for choosing the “smooth” stimulus in Set 1, would now be rewarded for choosing the “light” stimulus in Set 2. This shift always occurred across stimulus dimensions (texture and color). For Set 2, all rats performed 80

trials with the rewarded stimulus, regardless of how many trials were required to reach eight consecutive correct arm choices, as was the case in Set 1. Time to reach criterion was recorded for both sets.

At the end of Set 2 testing, the rats were returned to their home cages and all running wheels were locked for 7 days, so that none of the rats had access to voluntary exercise. All rats continued to undergo food deprivation during this time. A test one week later with the same rewarded stimulus as during Set 2 was conducted to analyze possible long-term effects of exercise between adolescent and adult rats. Specifically, this task measured long-term memory. The goal was for each rat to complete 32 trials. However, this phase of testing was not reinforced with sucrose pellets when the rat chose the correct arm. There was thus variability in rat motivation and number of trials completed. The lowest number of trials completed by a rat was 12 and so only the first 12 trials were analyzed for all the rats.

Data Analysis. Statistical analysis was conducted using SPSS 25.0. A one-way ANOVA was used to analyze time to complete 8 trials for habituation data from the day before Set 1. A 2 (Adolescent/Exercise, Adult/Exercise) X 14 (Running Day) repeated-measures ANOVA was used to analyze a Running Day main effect. Since the experiment was based on a 2 (adolescent or adult) x 2 (exercise or no exercise) factorial design, 2 x 2 ANOVA tests were used to compare groups on 4 dependent measures: trials to criterion for Sets 1 and 2 and time per trial for Sets 1 and 2. Set 2 time per trial was calculated by dividing the total time to complete Set 2 by the total number of trials. Perseverative responding in Set 2 was defined as choosing an arm that had been reinforced in Set 1 but was not reinforced in Set 2. Perseverative responding was evaluated across blocks by computing the percentage of correct choices from the two start arms, or “perseverative arm starts”, where choosing according to the Set 1 rule was no longer correct.

Non-persistent responding in Set 2 was defined as choosing an arm that continued to be reinforced in Set 2 after being reinforced in Set 1. Non-persistent responses were evaluated across blocks by computing the percentage of correct choices from the other two start arms, or “reinforcement start arms”, where choosing according to the Set 1 rule was still correct. Lastly, percent correct for the first 12 trials of the repeat Set 2 test were calculated. Percent correct was calculated by counting the number of correct arm choices divided by the total number of trials completed.

Results

Rats Removed from Study.

Prior to data analysis, 3 rats were removed from the study due to not eating pellets in Set 1, 1 rat was removed due to not reaching a criterion of 8 trials correct in Set 1 because the rat stopped moving in the maze, and 1 rat was removed because the rat stopped eating pellets during open maze habituation, subsequently not eating pellets in Set 1 or Set 2. This left us with a total of 43 rats used in data analysis. Final group numbers were 12 adolescent exercising rats, 9 adolescent non-exercising rats, 11 adult exercising rats, and 11 adult non-exercising rats.

Habituation Data.

Habituation data from the day before Set 1 were compared to check for any motivational differences between the exercising and non-exercising rats. During this session, rats completed 8 trials with one arm randomly baited on each trial. Time to complete the 8 trials was recorded. A one-way ANOVA revealed no difference in time (seconds) to complete 8 trials.

Voluntary Wheel Running Daily Distances.

Figure 5 depicts the amount of wheel running (in kilometers) for rats in the exercising group. A 2 (Adolescent/Exercise, Adult/Exercise) X 14 (Running Day) repeated-measures

ANOVA revealed a Running Day main effect for adults, $F(13,130)=2.95$, $p=0.001$. Unexpectedly, a Running Day main effect was not seen for adolescents, $F(13,143)=1.285$, $p=0.228$. Other studies analyzing this same measure have seen a Running Day main effect for both adolescent and adult rats.

Set 1: Trials to Criterion.

There was no difference between the adolescent exercise and no exercise groups and between the adult exercise and no exercise groups in number of trials to meet a learning criterion of 8 correct choices in a row. This was confirmed by a 2 (Adolescent vs. Adult) X 2 (Exercise vs. No Exercise) ANOVA. The age x exercise effect was not significant, $F(1,39)=0.08$, $p>0.05$, the age effect was not significant, $F(1,39)=1.24$, $p>0.05$, and the exercise effect was not significant, $F(1,39)=0.99$, $p>0.05$ (Figure 2).

Set 2: Trials to Criterion.

There was no difference between groups in number of trials to meet a learning criterion of 8 correct choices in a row. This was confirmed by a 2 (Adolescent vs. Adult) X 2 (Exercise vs. No Exercise) ANOVA. The age x exercise effect was not significant, $F(1,39)=1.01$, $p>0.05$, the age effect was not significant, $F(1,39)=0.08$, $p>0.05$; and the exercise effect was not significant, $F(1,39)=1.01$, $p>0.05$ (Figure 2).

Set 2: Time per Trial.

The time per trial measure for Set 2 was used to assess motivation and motor abilities. There was no difference between the groups. This was confirmed by a 2 (Adolescent vs. Adult) X 2 (Exercise vs. No Exercise) ANOVA. The age x exercise effect was not significant, $F(1,39)=2.89$, $p>0.05$, the age effect was not significant, $F(1,39)=0.02$, $p>0.05$, and the exercise effect was not significant, $F(1,39)=0.42$, $p>0.05$.

Set 2: Performance Across Trial Blocks on Perseverative Arm Start Trials.

On Set 2 “perseverative arm start” trials, the correct arm choice was the arm that was not reinforced in Set 1. All groups began Set 2 at less than chance on these trials (thus making many perseverative errors) and learned to shift across trial blocks. In an analysis of percentage correct across 10 blocks of 4 trials each, there was no difference between any of the groups (Figure 3). This was confirmed with a 2 (Adolescent vs. Adult) X 2 (Exercise vs. No Exercise) X 10 (Block) repeated measures ANOVA, which revealed a Block main effect, $F(9,351)=15.97$, $p<0.05$. No other effects were significant including age main effect, $F(1,39)=0.46$, $p>0.05$; exercise main effect $(1,39)=0.14$, $p>0.05$; block X age effect, $F(9,351)=0.65$, $p>0.05$; block X exercise effect, $F(9,351)=0.81$, $p>0.05$; and block X age X exercise effect, $F(9,351)=0.44$, $p>0.05$.

Set 2: Performance Across Trial Blocks on Reinforcement Arm Start Trials.

On Set 2 trials where the correct arm choice was the arm that was reinforced in Set 1, all groups began Set 2 at greater than chance (retaining the Set 1 rule) and increased correct performance across trial blocks. In an analysis of percentage correct across 10 blocks of 4 trials each, there were no differences across the groups (Figure 4). This was confirmed with a 2 (Adolescent vs. Adult) X 2 (Exercise vs. No Exercise) X 10 (Block) repeated measures ANOVA, which revealed a Block main effect, $F(9,351)=12.92$, $p<0.05$. No other effects were significant including age main effect, $F(1,39)=0.01$, $p>0.05$; exercise main effect $(1,39)=0.49$, $p>0.05$; block X age effect, $F(9,351)=0.37$, $p>0.05$; block X exercise effect, $F(9,351)=0.73$, $p>0.05$; and block X age X exercise effect, $F(9,351)=0.67$, $p>0.05$.

Set 2 Repeat: Percent Correct out of total trials.

The goal was for each rat to complete 32 trials but the total number of trials completed varied for each rat because this testing phase was not reinforced with sucrose pellets. The lowest

number of trials completed until the rat no longer ran in the maze was 12 and the highest completed was 36. Percent correct was thus calculated based on only the first 12 trials. A 2 (Adolescent vs. Adult) X 2 (Exercise vs. No Exercise) ANOVA was used to confirm that the age x exercise effect was not significant, $F(1,39) = 0.88, p > 0.05$, the age effect was not significant, $F(1,39) = 2.48, p > 0.05$, and the exercise effect was not significant, $F(1,39) = 0.29, p > 0.05$.

Discussion

In general, there is little known about exercise-related effects on cognitive flexibility, in either adolescents or adults. In the current experiment, there was no evidence that exercising adolescent rats (aged approximately 28-42 days old during exercise) had facilitated set-shifting in a T-maze compared to non-exercising adolescent rats. Exercising young adult rats (44-58 days old during exercise) also did not have any enhancements in set-shifting. We also did not see adolescent exercising rats having longer lasting cognitive benefits of exercise, given that the rats did not perform with higher accuracy in a repeat Set 2 test one week after termination of exercise. Both exercising adolescent and adult rats performed the same as non-exercising adolescent adult rats in Set 1, which was expected (Eddy et al., unpublished).

The current study was a follow up to Eddy et al. (unpublished). That study consisted of two separate experiments, one examining the effects of exercise on adolescent rats and the other examining the effects of exercise on young adult rats. They also incorporated the use of injections of either vehicle or methylphenidate (MPH; a dopamine transporter and norepinephrine transporter blocker) and hypothesized that it would have similar behavioral effects as exercise, with improvements in rodent attention and cognitive flexibility. Experiment 1 examined adolescent rats aged 30-44 days old during exercise (which was classified as early-to-middle adolescence) and Experiment 2 examined adult rats aged 44-58 days during exercise

(which was classified as late adolescence-to-pre-adulthood). This was approximately the same age of adolescent and adult rats used in the current study. We were able to replicate the lack of difference seen between exercise and non-exercise groups in discrimination learning (Set 1). However, Eddy et al. (unpublished) found an exercise-related facilitation in performance in Set 2 in adolescent exercising rats, which was not seen in the current study. There are, however, some differences between the two studies that may have contributed to the differences in the results. First, Eddy et al. (unpublished) consisted of two different experiments conducted at different times. This makes it difficult to directly compare performance between adolescent and adult rats in their study, and it also may have introduced variables that could have affected results (such as a different experimenter handling rats between experiments or different colony conditions). Second, it may be that daily injections, even of just vehicle, influenced the results of Eddy et al. (unpublished). It is possible that exercise mitigated the effects of the stress of the injections in adolescent rats, given that vehicle-injected exercising adolescent rats performed better than vehicle-injected non-exercising adolescent rats. In the current study, rats did not undergo injections of any kind.

Another discrepancy between Eddy et al. (unpublished) and the current study is that we observed a greater percent correct at the beginning of Set 2 for perseverative arm starts, ~40% compared to ~30%. Perseverative arm starts were Set 2 trials where the correct arm choice was the arm that was not reinforced in Set 1. This may be because our rats did not retain the Set 1 rule quite as well as in the previous study, so there was less interference with Set 2 performance. This interpretation is supported by our results with reinforcement arm starts. We observed a lower initial percent correct in reinforcement arm starts, ~50% compared to about ~60%. Reinforcement arm start trials represent Set 2 trials where the correct arm choice was the arm

that was reinforced in Set 1, and the percentages indicate that rats began Set 2 retaining the Set 1 rule, but slightly less well than in Eddy et al. (unpublished). Both the higher than expected perseverative percentage and lower than expected reinforcement percentage would suggest that our rats showed poorer retention of the Set 1 rule than in Eddy et al. (unpublished).

However, another study showed that exercise during adulthood improved performance in initial discrimination learning (Set 1) but not in set-shifting (Set 2) (Eddy et al., 2013). In that study, adult rats (65-79 days old when exercising) were given access to two weeks of either a locked or unlocked running wheel in their home cages and were then tested in a our T maze-based extra-dimensional set-shifting task. This is the same procedure used in the current experiment; however, the adult rats in Eddy et al. (2013) were older compared to the adult rats in the current experiment (approximately 44-58 days old while exercising). This suggests that there might be varying cognitive benefits of exercise based on age within adulthood, specifically younger adults or more mature adults.

There was also a discrepancy seen in the average running per day between studies. In Eddy et al. (2013), adult male rats ran upwards of 10-12 km per day, and in Eddy et al. (unpublished) both adolescent and young adult rats ran between 8-16 km daily for most of the exercising period (10 of the 14 exercising days). This is significantly more exercise than rats in the current study, with the average maximum running per day we observed not exceeding about 5 km in the 14 day exercise period for both adult and adolescent rats. We considered that we did not see significant improvement in set-shifting in exercising rats because they were not running as much as in other studies that have shown an exercise-associated improvement in Set 1 or Set 2 performance. To determine whether the amount of running influenced the number of trials to reach criterion (8 trials correct in a row), we examined the correlation between how much rats

ran on Day 14 of running wheel access and their trials to criterion. We did not find a correlation between the measures and thus can infer that the amount a rat runs may not significantly affect their performance on our set-shifting measure. We examined this across all rats ($r=0.113$, $p=0.469$) as well as across each age group separately, adult rats ($r=0.150$, $p=0.506$) and adolescent rats ($r=0.276$, $p=0.227$). It is also important to note that there was no Running Day main effect seen in exercising adolescent rats in the current study, whereas there was a Running Day main effect seen in adult exercising rats. This shows that adolescent rats were not increasing the amount they were running over the 14 days, whereas the adult rats were. This result is different than Eddy et al. (unpublished) as well as Eddy and Green (2017) where a Running Day main effect was seen in both groups. This shows that the adolescent rats in the current study were not running as much as was seen in prior studies, which could play a role in why we did not see a significant enhancement in set-shifting for exercising adolescent rats. Perhaps there is a certain amount that rats have to exercise before getting the cognitive benefits of exercise, and the adolescent rats in the current experiment did not reach that threshold.

Another study used an ABA renewal paradigm to determine the effects of exercise in adolescent and adult rats (Eddy & Green, 2017). Similarly to our study and Eddy et al. (unpublished), adolescent rodents were approximately 44 days old at the time of testing and adult rats were approximately 70 days old at the time of testing. As we did in the current study, Eddy and Green (2017) also gave all rodents access to locked or unlocked running wheels to control for environmental enrichment factors. They showed that exercising adolescent rats had reduced ABA renewal of extinguished instrumental responding compared to non-exercising adolescent rats but exercising adults did not differ from non-exercising adults. They also found a higher density of dendritic spines and a lower dendritic branch length in the mPFC of adolescent

exercising rats compared to adolescent non exercising rats, whereas there was no difference in these measures between adult exercising and non exercising rats. Compared to Eddy et al. (unpublished), there was no stressor in Eddy and Green (2017) where exercise might have mitigated stressor effects and caused the improvement in performance. Thus, we can infer that it may be the difference in tasks between our study and that of Eddy and Green (2017) that produced the difference in results.

There was also a discrepancy between our results and the results of a study comparing cognitive flexibility in adult rats after 12 days of exercise (Brockett et al., 2015). That study assessed performance of rats in several different discriminations, including an extradimensional shift (as we did), which is a task known to involve the mPFC and required the rats to discriminate between different digging media and/or textures covering digging containers. The extradimensional shift required rats to shift attention between media or texture covering the digging container between testing days. It was found that exercising rats seemed to have increased cognitive flexibility. However, Brockett et al., (2015) used a different task to measure cognitive flexibility than in our experiment, and it is unclear in their experiment whether the other discriminations, such as the intradimensional shift or reversal learning impacted the exercise-related improvement in extra-dimensional set-shifting. Their study also only used adult rats and did not clarify the weight or exact ages of the adults, and so it is unclear if their rats were young adult rats as in our study (44-58 days old) or if they were more mature adults (65-79 days old) such as in Eddy et al. (2013). Given that we do not know the ages of the adult rats in the study, we may not be to compare performance to our adult rats, which are classified as late adolescence-to-pre-adulthood according to Eddy et al. (unpublished). Another difference is that Brockett et al. (2015) housed rats in groups of 3 while we housed our rats in individual cages;

they also provided exercising rats with a running wheel in their cage but non exercising rats with no running wheel at all. In our study, we provided both groups with a running wheel (unlocked or a locked) to control for environmental enrichment factors. Lores-Arnaiz et al. (2007) found that exposure to an enriched environment during adolescence improved spatial learning and memory. This brings up the question as to whether we did not observe exercise-related effects in our study based on the fact that all of our rats might be considered “enriched”.

Brockett et al. (2015) also found that adult exercising rats that underwent the extradimensional set shifting task had enhancements in synaptic, dendritic, and astrocytic measures in the medial prefrontal cortex. The findings from this study as well as Eddy and Green (2017), who found that there were no differences in dendritic measures between adult exercising and non exercising rats, suggest that there are inconsistencies with the neurological changes found in adult mPFC as a result of exercise. However, the groups of rats in Brockett et al. (2015) could be differentiated into “enriched” versus “non-enriched” groups based on presence of a running wheel, whereas the rats in Eddy and Green (2017) only differed in whether they had access to the running wheels to exercise. Thus, it is possible that the changes seen in the more mature adult mPFC may be due to in enrichment rather than exercise. It may be that Eddy and Green (2017) did not see changes in the less mature adult mPFC because they did not have non-enriched rats, as there were in Brockett et al. (2015). These two studies also used different tasks than in the current study.

A potential future direction for the current research would be to perform histology to obtain data on astrocytic or dendritic changes between adolescent and adult exercising and non-exercising rats in an experiment directly comparing the groups, unlike in previous studies where the age groups were studied in separate experiments. This would allow us to determine if there

are morphological changes in the mPFC as a result of exercise when directly comparing adolescent and young adult rats. If there are no morphological changes that may lead us to assume that maybe the rats did not exercise enough to cause morphological changes in the brain, and that is the reason we are not seeing any behavioral changes. It is also possible that morphological changes as a result of exercise may only occur during specific ages, such as only during late adulthood compared to young adulthood.

In summary, the current study showed that voluntary exercise did not significantly improve performance in a set-shifting task in adolescent rats. However, it is still possible that exercise may have effects that were not seen in the current study for the reasons discussed. Our study used adolescent rats aged 28-42 days during exercise and adult rats aged 44-58 days during exercise. We were overall unable to replicate the results seen in Eddy et al. (unpublished), which also examined adolescent and adult exercising rats, but in two separate experiments and combined with daily injections. Given that the current experiment directly compared adolescent and adult rats in a 2 x 2 factorial design, our results provide evidence that exercise during adolescence or very young adulthood does not improve cognitive flexibility. However, given that other studies have shown effects of exercise in adolescents but not in adults, there is a need for further research into the differences between adult and adolescent benefits from exercise.

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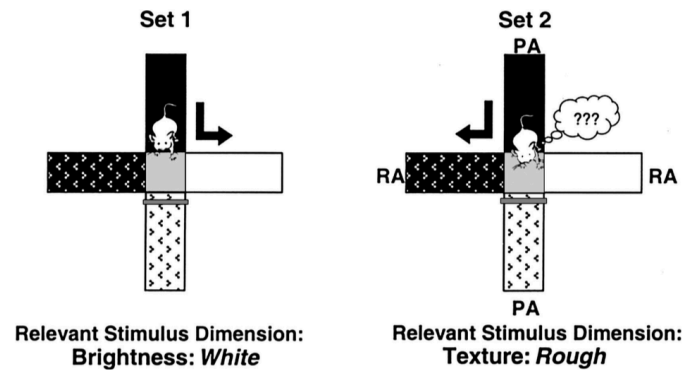


Figure 1. T maze-based extra-dimensional set-shifting task design. This shows that on the first day of testing (Set 1), the rat will be rewarded for the brightness dimension, specifically the white color. During testing the next day (Set 2), the rat is rewarded for an extradimensional shift. The rat is now rewarded for the texture dimension, specifically a rough texture.

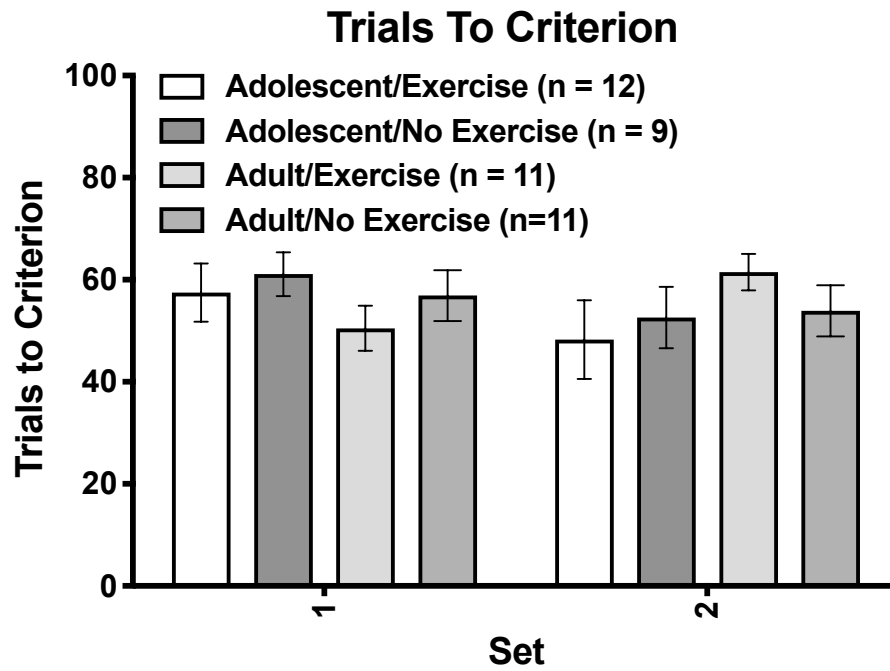


Figure 2. Extradimensional set-shifting in adolescent and adult rats is based on either exercise or no exercise. Trials to a criterion of 8 correct arm choices in a row as a function of group for Set 1 (initial discrimination) and Set 2 (extradimensional set-shift).

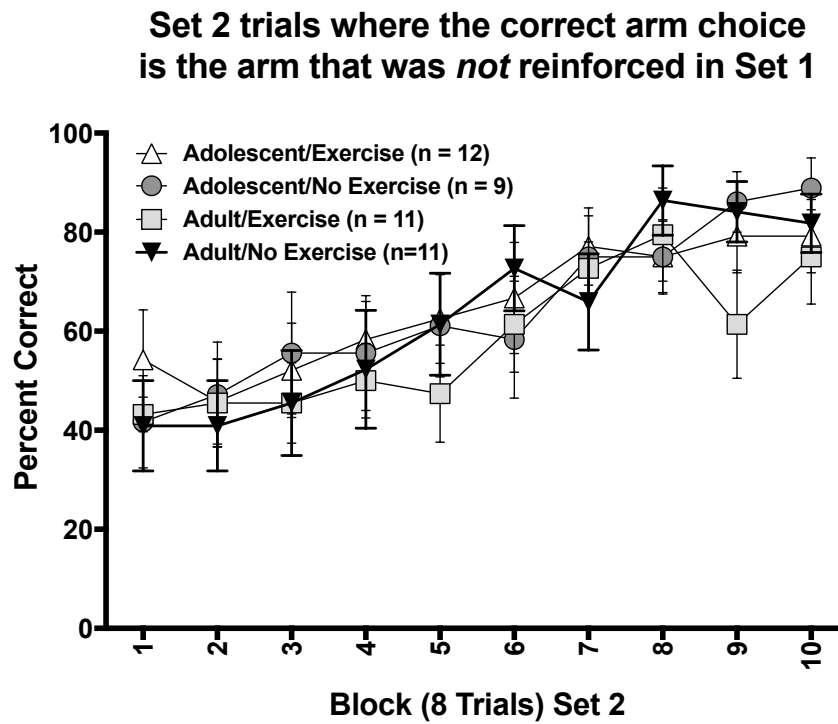


Figure 3. There was overall a reduction in perseverative errors across all groups. Percentage of correct arm choices as a function of group in each block of 8 trials in Set 2 in which the correct arm choice requires an extradimensional set-shift.

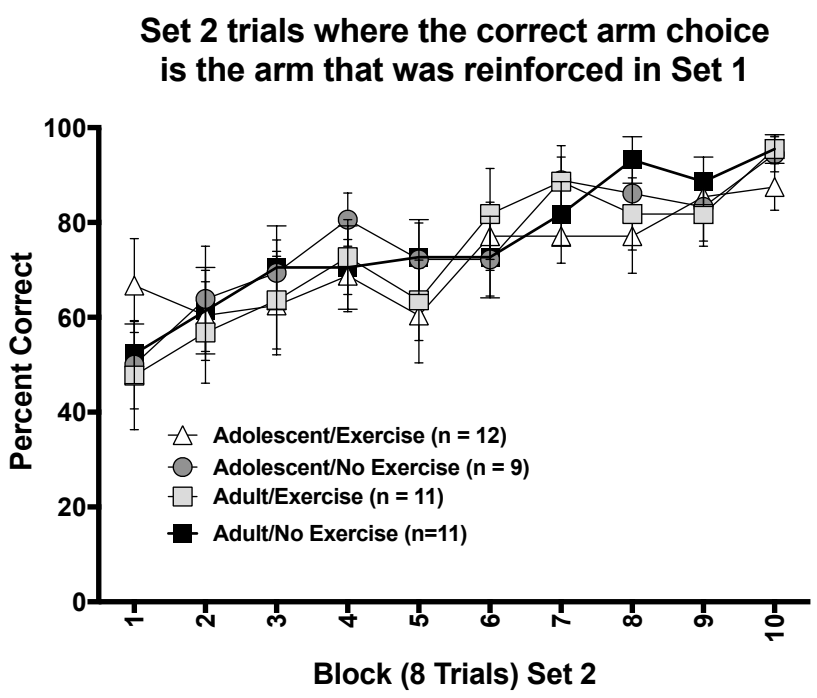


Figure 4. Percentage of correct arm choices as a function of group in each block of 8 trials in Set 2 in which the correct arm choice is the same as in Set 1.

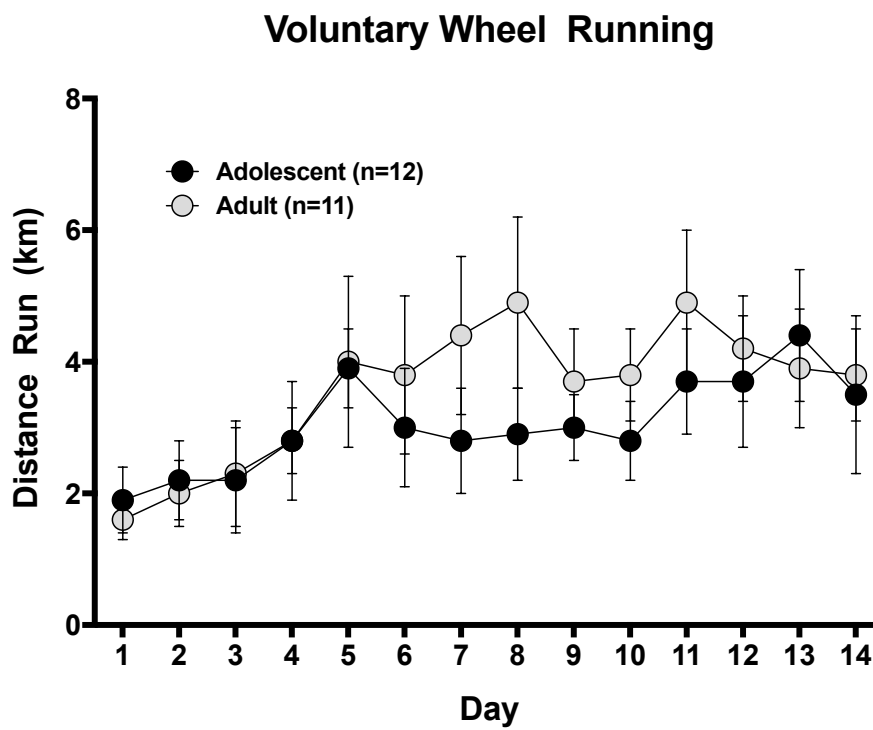


Figure 5. The daily amount of wheel-running exercise is similar in early-to-middle adolescence (30-44 days old) and late adolescence-to-young-adulthood (43-57 days old).