Differential Effects of Adolescent and Adult-initiated Exercise on Cognition and Hippocampal Neurogenesis

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Abstract

Adolescence is a critical period for postnatal brain maturation and thus a time when environmental influences may affect cognitive processes in later life. Exercise during adulthood has been shown to increase hippocampal neurogenesis and enhance cognition. However, the impact of exercise initiated in adolescence, on the brain and behavior in adulthood is not fully understood. The aim of this study was to compare the impact of voluntary exercise that is initiated during adolescence or early adulthood on cognitive performance in hippocampal-dependent and independent processes using both object-based and touchscreen operant paradigms. Adult (8 week) and adolescent (4 week) male Sprague Dawley rats had access to a running wheel (exercise) or were left undisturbed (sedentary control) for four weeks prior to behavioural testing and for the duration of the experiment. Results from touchscreen-based tasks showed that reversal learning was enhanced by both adult and adolescent-initiated exercise, while only exercise that began in adolescence induced a subtle but transient increase in performance on a location discrimination task. Spontaneous alternation in the Y-maze was impaired following adolescent onset exercise, while object memory was unaffected by either adult or adolescent-initiated exercise. Adolescent-initiated exercise increased the number of hippocampal DCX cells, an indicator of neurogenesis, and also promoted the complexity of neurites on DCX cells, a key process for synaptic integration, to a greater degree that adult-initiated exercise. Together the data here show that exercise during the adolescent period compared to adulthood differentially affects cognitive processes and the development of new hippocampal neurons in later life.

Introduction

The positive effects of exercise on learning and memory is well established (for reviews, see (Gomez-Pinilla & Hillman, 2013; Voss, Vivar, Kramer, & van Praag, 2013)). Specifically, voluntary exercise in adulthood has been shown to enhance hippocampal-dependent cognition, such as spatial learning (Anderson et al., 2000; van Praag, Shubert, Zhao, & Gage, 2005) and contextual fear conditioning (Baruch, Swain, & Helmstetter, 2004; Kohman et al., 2012) in both rats and mice. Moreover, other forms of learning, such as cognitive flexibly, a prefrontal cortex-mediated processes is also increased following exercise in adult rats and mice (Brockett, LaMarca, & Gould, 2015). Exercise-induced enhancement in cognitive function is often associated with significant changes in the neurocircuitry involved in learning and memory, such as the hippocampus and prefrontal cortex (Brockett et al., 2015; Creer, Romberg, Saksida, van Praag, & Bussey, 2010). In particular, exercise in adulthood has been shown to be a potent stimulator of hippocampal neurogenesis, a form of structural plasticity that occurs in the dentate gyrus (DG) of the hippocampus (van Praag, Christie, Sejnowski, & Gage, 1999). Indeed, it is hypothesized that the beneficial effects of exercise on hippocampal-dependent cognition is due, in part, to its pro-neurogenic capacity (Clark et al., 2008; Ji et al., 2014). Adult hippocampal neurogenesis has been shown to be necessary for cognitive processes such as spatial memory and contextual memory, as well as the ability to discriminate between similar memories (Frankland, Köhler, & Josselyn, 2013; Rola et al., 2004; Saxe et al., 2006; Snyder, Hong, McDonald, & Wojtowicz, 2005). This process of pattern separation encodes memories in a discrete non-overlapping fashion, and without such a process, memory recall would suffer high interference from similarly encoded memories (Kent, Hvoslef-Eide, Saksida, & Bussey, 2016). Pattern separation has been repeatedly associated with hippocampal neurogenesis (Aimone, Deng, &

Gage, 2011; Besnard & Sahay, 2016; Revest et al., 2009; Sahay, Wilson, & Hen, 2011). Indeed, several weeks of running wheel exercise in adult mice has been shown to enhance pattern separation through upregulation of hippocampal neurogenesis (Creer et al., 2010). In recent years, novel cognitive tests have been developed to tease apart the relationship between hippocampal neurogenesis and cognitive function. One such approach has been the development of touchscreen-based tests which allow for increased translation of pre-clinical research findings, and thus help to bridge the species divide (Horner et al., 2013; Oomen et al., 2013). The majority of studies to date, using both object and touchscreen-based approaches to assess cognitive function, have focused on the effects of exercise in adulthood, when the neural circuitry underling learning and memory is physiologically mature. However, the impact of exercise on cognitive processes during key developmental periods when the hippocampus and prefrontal cortex are still undergoing maturation is yet to be fully explored (Hueston, Cryan, & Nolan, 2017).

Adolescence is a sensitive period for maturation of the hippocampal circuitry, during which time an increase in the number of granule cells and dendritic arbors occurs, as well as increased synaptic pruning and an overall increase in the volume of the hippocampal layers (Bayer, 1982; Fuhrmann, Knoll, & Blakemore, 2015; Hueston et al., 2017; Schneider, 2013; Sousa, Madeira, & Paula-Barbosa, 1998). In order for these new neurons to process information, extensive neurite arborization occurs so that neurons are capable of receiving and integrating complex synaptic inputs. Thus, adolescence may be a critical period during which alterations in hippocampal function may result in organizational effects which last throughout adulthood (Blakemore & Choudhury, 2006; Curlik, Difeo, & Shors, 2014; Fuhrmann et al., 2015; Schneider, 2013; Spear,

2004). The prefrontal cortex also undergoes significant maturation during the adolescent period which continues into early adulthood, with increases in synaptic pruning and myelination (Blakemore & Choudhury, 2006; Casey, Getz, & Galvan, 2008; Giedd et al., 1999; Spear, 2013). These developmental processes allow for the strengthening and fine tuning of connections between the hippocampus and prefrontal cortex and are thought to underlie the emergence of cognitive functions which typically develop in adolescence, such as response inhibition and cognitive flexibility (Selemon, 2013; Spear, 2013). We propose that exercise during adolescence may capitalize on the peak in neural plasticity during this period. Thus, the aim of this study was to determine the impact of voluntary exercise initiated at adolescence on hippocampal neurogenesis, neurite arborization and cognitive performance in hippocampal neurogenesis-dependent and independent tasks in adulthood.

Methods

Animals and Experimental Design

Adult (8 week) and adolescent (4 week) male Sprague Dawley rats were obtained from Envigo Laboratories (The Netherlands). All rats were paired housed in standard housing conditions (temperature 22 ± 1°C, relative humidity 50%) with a 12:12 hour light-dark cycle (0730-1930) and had *ad libitum* access to food and water. All experiments were conducted in accordance with the European Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork (AE19130/1019). Two independent cohorts of rats (adult and adolescent) were pair housed in either standard housing (control group n = 10 for each cohort) or with continuous access to a running wheel (Techni plast, UK) (exercise group n = 10 for each cohort) for four weeks prior to behavioral testing (Figure 1). Animals were pair housed in order to avoid any potential social isolation stress effects of single housing on hippocampal neurogenesis (Leasure & Decker, 2009; A. M. Stranahan, Khalil, & Gould, 2006). We saw that pair housed animals were able to run simultaneously on the running wheel and they often shared the wheel during the course of the study. Access to a running wheel was maintained for the duration of the experiment (11 weeks for the adult-initiated exercise and 12 weeks for the adolescent-initiated exercise), and running distance (km) per 24h was recorded. Voluntary running wheel activity is a well-adopted exercise paradigm of experience-based change in synaptic plasticity that simulates aspects of voluntary human behaviour (Molteni, Ying, & Gomez-Pinilla, 2002). All rats with access to running wheels ran an average of 2.5 (km) per 24h for the duration of the study. The adult-initiated exercise cohort steadily increased the amount of running throughout the study (Figure 2), whereas the adolescent-initiated cohort initially

increased the amount of running for the first 7 weeks after which the distanced travelled decreased (Figure 2). It is possible that the fluctuation in running performance may stem from the need to food restrict animals for the touchscreen operant training. Rats were assessed by the following behavioral tests: spontaneous alternation in the Y-maze, novel object recognition using object-based tasks, and location discrimination and reversal learning using touchscreen operant-based tasks. Both cohorts of rats (control and exercise) were sacrificed three weeks after completion of the behavioral tasks and brain tissue was collected for immunohistological analysis of hippocampal neurogenesis and neurite arborization (Figure 1).

Touchscreen Pre-training

Touchscreen chambers consisting of a rectangular operant box with grid flooring, overhead light, a touchscreen, and food hopper were used (Med Associates, USA). Following three days of food restriction (90% of free feeding weight), rats were trained to use the touchscreens in 5 stage's as previously described (Horner et al., 2013). Briefly, in stage 1, rats were habituated to the touchscreen chambers and food pellets for 30 min each day for two days. During stage 2, a relationship between the visual stimuli ((images of white squares), Figure 3A, D) and a food reward was introduced. Stimuli were presented on the touchscreen for 30 seconds, following which a pellet reward was delivered. Each displayed image and reward collection pair was referred to as a trial. The inter trial interval for stage 2 and all subsequent stages was 20s. If the image was touched by the rat, a three pellet reward was delivered to encourage future responses to the displayed image. Once the rat had completed 60 trials within 60 minutes the animal advanced to the next training stage. During stage 3, visual stimuli were presented on the

touchscreen until a response was made, upon which a reward was presented. Again, once the rat had completed 60 trials within 60 minutes the animal advanced to the next training stage. Stage 4 was similar to stage 3 with the addition of a trial initiation step where rats had to initiate the onset of each trial with a nose-poke into the reward delivery magazine. Again, once the rat had completed 60 trials within 60 minutes the animal advanced to the next training stage. During stage 5, a penalty (5 second time-out period with house light on) was introduced for touches to an area of the touchscreen that was not displaying the image, thus shaping the animals' response to the visual stimuli only. In stage 5, criterion was 100 trials with ≥ 80% correct on two consecutive sessions in 60 minutes.

Location Discrimination Training

Location discrimination was assessed as described by Oomen et al. (2013). Rats were initially trained on an intermediate separation, consisting of two response image locations with an intermediate inter-stimulus distance (5cm); one image location was reinforced (CS+) and the other was punished (CS-). Rats were required to obtain 7 correct trials out of 8. The reinforced location was then reversed and the animal was again required to learn the new reward contingency (7 correct trials out of 8); this was referred to as a reversal. The intermediate separation was continued until the animal was able to attain the initial location-reward contingency, as well as the subsequent reversal within one session (60 minutes) in three out of four consecutive sessions. Upon successful completion of training, rats advanced to the location discrimination task.

Touchscreen Location Discrimination Testing

Following successful completion of the intermediate training, rats proceeded to the location discrimination test. The location discrimination test consisted of a large separation (large inter-stimulus distance, 8cm) and a small separation (small inter-stimulus distance, 1cm). The trial structure of these sessions were identical to the intermediate trials as described above; rats were allowed unlimited trials in 60 minutes to complete as many reversals as possible (7 correct trials out of 8). Rats were exposed to 2 sessions of each separation (large or small, Figure 3A and D) per block, with each rat completing 3 blocks of trials (i.e. 6 sessions in total for each large and small separation). Both the starting separation (small or large) and reward location (left or right) were counterbalanced between groups. The number of trials to complete the first reversal was recorded, as well as the total number of reversals completed within the 60-minute session.

Spontaneous Alternation in the Y maze

Spontaneous alternation behaviour is the tendency of rodents to alternate their exploration of maze arms (such as those of the Y maze) and is used as a measure of hippocampal-dependent working memory (Hughes, 2004). The Y maze consisted of three arms 120° from each other (40 x 10 x 20 cm; made in house). The protocol was adapted from Senechal, Kelly, Cryan, Natt, and Dev (2007). Each animal was placed into the first arm of the maze facing the wall, and allowed to explore the maze for five minutes. The number and order of arm entries were recorded. An arm entry was defined as all four paws entering into the arm (four paw criteria). An alternation was determined as the number of consecutive entries into the three maze arms. Alternations were then divided by the total number of entries during the five-minute test period. The percentage of alternations was calculated as % = Alternations/(Entries-2).

Novel Object Recognition

Novel object recognition, a hippocampal-perirhinal cortex dependent task, was assessed as described by Bevins and Besheer (2006). On day 1 the rats were habituated to the testing arena (rectangle arena) for a 10-minute exploration period. On day 2, two identical objects were positioned on adjacent corners approximately 5 cm from each wall of the arena and each animal was introduced for a 10-minute exploration period. Rats were then placed directly back into their home cages. After a three-hour inter-trial interval, one familiar object was replaced with a novel object, and each animal was introduced for a five-minute exploration period. Object exploration was defined when the animal's nose came within a 2 cm radius of the object. The testing arena and objects were cleaned with a 50% alcohol solution. Video recordings were made to allow for manual scoring of object exploration. Object discrimination was calculated as the time spent exploring the novel object divided by the total time spent exploring both objects.

Tissue processing and immunohistochemistry

On completion of the behavioural testing, rats were deeply anesthetised with sodium pentobarbital and then transcardially perfused with 4% paraformaldehyde. Brains were post-fixed for 24 hours in 4% paraformaldehyde, transferred to 30% sucrose until full penetration of cryoprotectant occurred, snap frozen in liquid nitrogen and stored at -80°C. Coronal sections through the DG were collected onto slides at 40 µm thickness in a 1:6 series.

Non-specific antibody binding was blocked using 10% normal donkey serum (NDS) in a solution of PBS with 0.3% Triton–X100 and tissue sections were incubated with goat anti-DCX (DCX, Goat Polyclonal, Santa Cruz sc-8066, 1:100). Sections were then incubated with a

secondary antibody (biotinylated rabbit anti-goat) and DCX-positive cells were visualised with a solution of 3,3'-diaminobenzidine (DAB). A cresyl violet counterstain was applied, and sections were washed, mounted, and coverslipped with DPX mounting media.

Image analysis, cell quantification and morphology

Images were obtained using an Olympus BX 53 Upright Microscope (BioSciences Imaging Centre, Department of Anatomy and Neuroscience, UCC) and analysed at 20x magnification with bright field. A modified stereological approach was performed to estimate the number of DCX-positive cells. Systematic random sampling was employed through the whole DG by counting cells on both hemispheres of each section in 1:6 series (240 μm apart). The total number of cells were summed and divided by the number of sections analysed for each animal, thus expressed as a number of DCX-positive cells per section. Cell quantification was performed using the image processing software package, ImageJ v 1.43m. Neurite length, the number of neurite branch points and the number of neurites per DCX-positive cell were analysed in the DG from rats that underwent adult-initiated exercise (n = 5) and adolescent-initiated exercise (n = 4). Ten randomly selected neurons were sampled per animal based on their having minimal overlap with neurites of adjacent neurons, thus there were 40-50 neurons analysed per group. Neurons were imaged at 100X magnification on an Olympus BX40 microscope and each was traced using *Camera Lucida (*Wollaston, 1807*).* The tracings were scanned onto a personal computer and analyzed using the Neuron J plug-in for Image J. Total neurite length was measured in pixels by a blinded observer and converted to μm using a scaled micrometer and Image J (Meijering et al., 2004). The extent of neurite branching was determined by counting the number of neurite branch points per neuron, and the number of processes per DCX-positive cell

was also quantified manually. The DG was also segregated into dorsal and ventral regions; dorsal -1.8 to -5.2 mm from bregma and the ventral from coordinates 5.2 to -6.7 mm, and cells in each segregation were quantified to determine any differences in neurogenesis and neurite complexity between the regions.

Statistical analyses

All data were analysed using SPSS statistical software (SPSS, Chicago, IL). Behavioural data (n = 8-10), neurite complexity (n = 4-5) and the number of DCX-positive cells (n = 3-4) were graphed as means +SEM. Data were analysed by Student's t-test or repeated measures ANOVA as described below. An alpha level of 0.05 was used as criterion for statistical significance, and probability levels quoted for non-significance. Standard errors of the mean (SEM) were used with all graphical output.

Results

Adolescent-initiated exercise preferentially promoted performance in a location discrimination task

No effect of exercise during adulthood was observed in both the large and small separation condition of the location discrimination task [$F(1, 36) = 0.50$, $p > 0.05$; Figure 3B], [$F(1, 36) = 0.76$, $p > 0.05$; Figure 3E], respectively. Similarly, there was no effect of exercise during adolescence on location discrimination in the large separation task [$F(1, 38) = 0.71$, $p > 0.05$; Figure 3C]. While there was no overall effect of exercise during adolescence on small separation task [$F(1, 38) = 1.71$, $p > 0.05$; Figure 3F], when t-tests at each of the 3 trial blocks were performed, there was a non-significant trend for adolescent exercise to increase performance in the second trial block [$t(38) = 1.84$, $p = 0.07$; Figure 3F].

Adolescent and adult-initiated exercise enhanced location discrimination reversal learning

There was a significant effect of adult-initiated exercise on reversal learning in the large separation condition, [$F(1, 36) = 14.52$, $p < 0.001$; Figure 4B]. Pairwise comparison indicated that rats beginning exercise in adulthood outperformed sedentary control adult rats in reversal learning in the large separation discrimination task during trial block 2 ($p < 0.001$) and 3 ($p < 0.001$), (Figure 4B). Likewise, in the small separation condition, there was a significant effect of adult exercise on reversal learning [$F(1, 36) = 5.97$, $p < 0.05$; Figure 4E]. Pairwise comparison indicated that rats beginning exercise in adulthood outperformed sedentary control adult rats in reversal learning in the small separation discrimination task during trial block 3 ($p < 0.01$), (Figure 4E). In the large separation condition, there was a non-significant trend of adolescent-initiated exercise to enhance reversal learning [$F(1, 38) = 2.31$, $p = 0.13$; Figure 4C]. A pairwise

comparison indicated that adolescent-initiated exercise outperformed sedentary control counterparts in reversal learning in the large separation discrimination task during trial block 3 ($p < 0.01$), (Figure 4C). Likewise, in the small separation condition, there was a significant effect of adolescent exercise on reversal learning [$F(1, 38) = 17.39$, $p < 0.0001$; Figure 4F]. A pairwise comparison indicated that rats beginning exercise in adolescence outperformed sedentary control counterparts in reversal learning in the small separation discrimination task during trial block 1 ($p < 0.05$) trial block 2 ($p < 0.001$), and trial block 3 ($p < 0.01$) (Figure 4F).

Adolescent-initiated exercise impaired spontaneous alternation in the Y maze but had no effect on novel object recognition

Exercise initiated during adulthood had no effect on spontaneous alternation in the Y-maze, a test of working memory [$t(18) = 0.97$, $p < 0.05$; Figure 5A]. However, rats that began exercise in adolescence showed impaired spontaneous alternation compared to sedentary control rats [$t(18) = 3.07$, $p < 0.01$; Figure 5B].

The novel object recognition memory test, a measure of short-term memory, was unaffected by exercise which began either in adulthood [$t(16) = 0.14$, $p < 0.05$; Figure 5C] or adolescence [$t(18) = 0.34$ $p < 0.05$; Figure 5D].

Adolescent-initiated exercise preferentially increased hippocampal neurogenesis compared to adult-initiated exercise

There was no change in the number of DCX-positive cells in the DG as a result of exercise initiated during adulthood [$t(6) = 1.13$, $p > 0.05$; Figure 6A], nor was there an effect within the dorsal [$t(6) = 0.84$, $p > 0.05$; Figure 6B] or ventral hippocampus [$t(6) = 1.97$, $p > 0.05$; Figure 6C]. However, adolescent-initiated exercise induced a significant increase in the number of DCX-positive cells in the DG [$t(4) = 2.87$, $p < 0.05$; Figure 6E]. This effect appeared to be driven by increases within the dorsal [$t(4) = 3.36$, $p < 0.05$; Figure 6F], but not the ventral hippocampus [$t(4) = 1.94$, $p > 0.05$; Figure 6G].

Exercise beginning in adulthood induced a significant increase in the neurite length of DCX-positive cells within the dorsal [$t(8) = 4.24$, $p < 0.01$; Figure 7A] and ventral hippocampus [$t(8) = 3.31$, $p < 0.05$; Figure 7A] as well as in the number of neurite branch points within both the dorsal [$t(8) = 2.57$, $p < 0.05$; Figure 7B] and ventral hippocampus [$t(7) = 2.55$, $p < 0.05$; Figure 7B], and the number of neurites per DCX-positive cell within the dorsal [$t(8) = 3.59$, $p < 0.01$; Figure 7C], but not ventral hippocampus [$t(7) = 2.20$, $p > 0.05$; Figure 7C]. Adolescent-initiated exercise increased the neurite length within the dorsal [$t(6) = 5.66$, $p < 0.01$; Figure 7D], but not the ventral hippocampus [$t(6) = 2.55$, $p > 0.05$; Figure 7D], although this approached significant it did not reach the .05 level. The number of neurite branch points was also significantly increased following adolescent exercise within the dorsal [$t(6) = 4.49$, $p < 0.01$; Figure 7E], but not ventral hippocampus [$t(5) = 2.00$, $p > 0.05$; Figure 7E] as well as the number of neurites per DCX-positive cell in the dorsal [$t(6) = 5.42$, $p < 0.01$; Figure 7F], but not the ventral hippocampus [$t(5) = 2.5$, $p > 0.05$; Figure 7F]. Adult-initiated exercise induced a 0.3-fold

increase in neurite length, 0.4-fold increase in the number of neurite branch points and a 0.4-fold increase in the number of neurites per DCX-positive cell in the dorsal hippocampus. However, the degree of change induced by adolescent-initiated exercise was much greater; a 0.7-fold increase in neurite length, a 1.3-fold increase in the number of neurite branch points and a 1-fold increase in the number of neurites per DCX-positive cell in the dorsal hippocampus.

When the complexity of neurites on DCX-positive cells in the dorsal hippocampus were directly compared between animals that began exercise in adulthood or adolescence, there was no significant difference in neurite length [$t(7) = 1.09$, $p > 0.05$; Figure 8A]. However, there was a significant increase in the number of neurite branch points [$t(7) = 2.70$, $p < 0.05$; Figure 8B] and the number of neurites per DCX-positive cell [$t(6) = 3.57$, $p < 0.01$; Figure 8C] following adolescent-initiated exercise.

Positive correlation between neurite length and cognitive flexibility as a result of exercise during adolescence

There was a significant positive correlation between the neurite length of DCX-positive cells in the dorsal hippocampus and performance in reversal learning during the small separation condition in rats that began exercise in adolescence [$r = 0.898$, $n = 8$, $p < 0.01$; Figure 9C]. This was not observed in rats that began exercise in adulthood [$r = 0.270$, $n = 8$, $p > 0.05$; Figure 9A]. No correlation was observed between neurite length and reversal learning performance in the large separation condition; adult-initiated exercise [$r = -0.098$, $n = 8$, $p > 0.05$; Figure 9B] and adolescent-initiated exercise, [$r = 0.554$, $n = 8$, $p > 0.05$; Figure 9D].

Discussion

This study revealed a differential effect of adolescent and adult-initiated exercise on cognition and neurogenesis in adulthood. Exercise that began in adolescence had a subtle effect on pattern separation, whereas both adult and adolescent-initiated exercise enhanced reversal learning in the touchscreen location discrimination task. Moreover, both adult and adolescent-initiated exercise increased the complexity of neurites on new born neurons in the DG, although exercise beginning in adolescence induced a greater fold increase in neurite complexity compared to exercise beginning later in life. Moreover, only adolescent-initiated exercise increased the number of these immature neurons in the DG.

The touchscreen location discrimination task was used to measure pattern separation. Performance in location discrimination during the small separation condition (i.e. when the images are close together and therefore have high contextual overlap) has been suggested to be a measure of pattern separation and to be sensitive to changes in hippocampal neurogenesis (Clelland et al., 2009; Creer et al., 2010; Oomen et al., 2013). The results of the current study indicated that exercise initiated in adulthood did not affect location discrimination during either the small or large separation conditions. Similarly, there was no difference in performance following exercise that began in adolescence in the large separation condition. However, there was a non-significant trend of enhanced performance during the small separation condition following adolescent-initiated exercise, although this mild effect was transient as both exercise and control animals performed similarly in the final training block. Most work to date on exercise-induced effects on pattern separation has been reported from experiments using adult or

aged rodents. Prolonged (ten weeks) of voluntary exercise enhanced performance during the small separation condition (i.e. when the images are close together and therefore have high contextual overlap) in the touchscreen operant chamber in aged mice (Creer et al., 2010). Likewise, voluntary exercise has been shown to increase pattern separation using a fear conditioning paradigm (in which contextual overlap varied between fear conditioning chambers) in aged (17 months old) mice (Wu et al., 2015). Studies using adult rodents have shown that forced treadmill exercise for six weeks increased pattern separation assessed in the radial arm maze (where conditions of either high or low spatial and contextual overlap were created between the sample arm and the choice arm) in adult mice (So et al., 2017). However, our results from the touchscreen operant chamber reveal that exercise during adulthood did not affect pattern separation. The difference in results we report may be due to methodological, species or age differences. Creer et al. (2010) initially trained the aged mice to use the touchscreen before the exercise regime began, while in the present study exercise began at the same time as touchscreen training. Moreover, it is possible that the exercise-induced increase in performance is more readily observed in an aged animal compared to healthy adult, due to an overall age-related decline in cognitive ability. Although exercise did not affect performance during touchscreen training in this study (data not shown), it is possible that transient exercise-induced changes in hippocampal functioning were lost after several weeks of operant training and once rats had reached criteria for location discrimination testing. Furthermore, there may be a ceiling effect in discrimination performance where optimal learning by healthy rats leaves no capacity for exercise-induced enhancements (Griffin, Bechara, Birch, & Kelly, 2009). Increasing the task difficulty (i.e. reducing the inter-stimulus distance) may help to overcome potential ceiling effects and improve task sensitivity to subtle and transient enhancements in performance of rats.

To date, no reports have been published on adolescent-initiated exercise on pattern separation despite the heightened plasticity that occurs in the hippocampus during this period of the lifespan. Interestingly, the subtle yet non-significant effect following adolescent-onset exercise that we observed in the current study was coupled with an increase in the number of immature neurons and the complexity of their neurites. Indeed, performance in the small separation condition of the touchscreen location discrimination task has previously been associated with increased survival of hippocampal new born neurons, albeit in aged mice (Creer et al., 2010).

Adult and adolescent-initiated exercise enhanced reversal learning, a hippocampal prefrontal cortex-mediated behaviour, which was assessed using the touchscreen location discrimination task and measured by the number of times the rat could switch between reward locations (i.e. reversals). Previous work has shown that running wheel exercise (two weeks) during adulthood in rats facilitated performance in an attentional set-shifting task, a measure of cognitive flexibility (Brockett et al., 2015). However, despite the dominant role of the prefrontal cortex in adolescent brain development and behaviour, to date no data have been reported to show the impact of adolescent-initiated exercise on prefrontal cortex-mediated cognitive flexibility. Exercise-induced enhancement of cognitive flexibility has previously been linked with structural changes in the hippocampus and prefrontal cortex in adulthood. Specifically, two weeks of running wheel exercise increased the spine density and spine length of neurons in the medial prefrontal cortex as well as inducing an increase in protein levels of the synaptic plasticity markers synaptophysin and PSD-95in the orbitofrontal cortex of adult rats (Brockett et al., 2015). Moreover, cognitive flexibility may also be modulated by exercise-induced increases in hippocampal plasticity which in turn alters hippocampal-medial prefrontal cortex projections and

thus influences executive function processes, such as cognitive flexibility (Burghardt, Park, Hen, & Fenton, 2012; Garthe, Behr, & Kempermann, 2009).

Spontaneous alternation in the Y-maze, a hippocampal-dependant spatial working memory task (Hughes, 2004), was unaffected by adult-initiated exercise, while adolescent-initiated exercise impaired spontaneous alternation behaviour. Previous studies have reported contradictory findings of exercise on working memory. Prolonged (seven weeks) running wheel exercise has been shown to improve spatial working memory in the radial arm maze in rats (Alomari, Khabour, Alzoubi, & Alzubi, 2016; Anderson et al., 2000), whereas, spatial working memory in the T-maze has been shown to be unaffected following one week of forced exercise in rats (Acevedo-Triana, Rojas, & Cardenas, 2017). The differences may also be due to the specific behavioral tasks used to measure spatial working memory i.e. spontaneous behavior versus training/reward directed behavior. Thus, exercise may enhance spatial working memory when the task is sufficiently challenging, such as in the radial arm maze where a correct response requires several days of training compared to one-time spontaneous exploration in the Y-maze. Moreover, exercise beginning earlier in life may not convey a global enactment of cognitive processes and in fact may negatively affect certain spontaneous behaviors in later life.

Novel object recognition, a hippocampal and entorhinal cortex-dependent processes, was unaffected by exercise that began in adulthood or adolescence. Findings from previous studies investigating the effects of exercise on object recognition have been varied. Studies have demonstrated an enhancement of object discrimination following either one week of forced exercise in adult rats (Griffin et al., 2009) or three and four weeks of running wheel exercise in adult rats (Bolz, Heigele, & Bischofberger, 2015; Hopkins & Bucci, 2010), whereas, two or three

weeks of running wheel exercise had no effect on object recognition in adult rats (Brockett et al., 2015) or adult mice (Bolz et al., 2015). This may be due to the fact that differences in the inter-trial interval between the sample and test phase have been shown to differentially recruit the hippocampus or perirhinal/ entorhinal cortex processes during object recognition (Cohen & Stackman, 2015; Hammond, Tull, & Stackman, 2004). Thus, tasks with a longer inter-trial interval (e.g. 24h) such as reported by (Bolz et al., 2015; Griffin et al., 2009; Hopkins & Bucci, 2010) detected an exercise-induced enhancement in discrimination, while tasks with a shorter inter-trial interval (a few hours), such as employed in the current study and by others (Bolz et al., 2015; Brockett et al., 2015) did not detect an exercise-induced effect. Indeed, Bolz et al. (2015) demonstrated that three weeks of voluntary exercise enhanced object discrimination following a 24 hour inter-trial interval, but did not impact object discrimination after a 1.5 hour inter-trial interval in adult rats.

Exercise that began in either adulthood or adolescence increased the dendritic complexity of immature neurons (DCX-positive cells) in the dorsal hippocampus. This is line with previous work showing that voluntary running wheel exercise for two months (Alexis M. Stranahan, Khalil, & Gould, 2007) and two to three weeks (Eadie, Redila, & Christie, 2005) increased the dendritic length and the number of dendritic spines of granule neurons in the DG of adult rats, although these studies report effects within the total hippocampus (i.e both dorsal and ventral). Interestingly, a recent report has demonstrated that as little as one week of running wheel exercise induced an increase the arborization of immature adult born granule cells as measured by total dendritic length and the number of dendritic branch points in new granule cells in the dorsal hippocampus of adult mice (Sah, Peterson, Lubejko, Vivar, & van Praag, 2017).

However, behavioral correlates were not assessed in this study and so it would be interesting to investigate the relationship between exercise-induced dendritic arborization of new hippocampal neurons and the emergence of any potential cognitive changes in terms of duration of exercise. Dostes et al. (2016) has reported that the effect of exercise on neuronal morphology is activity-dependent in that mice given unlimited (24 hour) access to a running wheel for three weeks showed a greater increase in dendritic complexity in the hippocampus compared to mice with limited (3 hour) running wheel access. Another factor to be considered in exercise-induced changes in cognition as a function of neurite complexity of new neurons is the age at which exercise is initiated. This is borne out in the results of the present study which reveal that exercise initiated in adolescence had a greater fold increase in complexity of neurites on new neurons compared to exercise that began in adulthood. Moreover, there was a positive correlation between the neurite length of new neurons and performance in reversal learning during the small separation condition in rats that began exercise in adolescence which was not observed in rats that began exercise in adulthood.

The hippocampus is functionally subdivided along the septotemporal axis into a dorsal and ventral region, which are associated with distinct behaviours (Fanselow & Dong, 2010; Moser & Moser, 1998). The process of spatial memory is generally associated with the dorsal hippocampus, while the ventral hippocampus is associated with emotional behaviour, particularly cued fear learning and anxiety-related behaviours (Bannerman et al., 2004). Indeed, the data presented here demonstrated that adolescent-initiated exercise increased neurite complexity of dorsal hippocampal DCX-positive cells to a greater degree than adult-initiated exercise, which aligns with the enhanced performance in a spatial-mediated location

discrimination task by these animals. Therefore, future work may investigate the impact of exercise along the septotemporal axis of the hippocampus during key developmental periods and how it relates to dorsal-ventral hippocampal processes, such as spatial learning and emotional regulation in later life.

Surprisingly, we observed that exercise that began during adolescence increased the number of immature neurons in the DG in adulthood while exercise initiated during adulthood did not. Furthermore, the results indicated that the effects of exercise on DCX-positive cells was driven by increases within the dorsal hippocampus. Previous reports have demonstrated the pro-neurogenic effect of exercise during adulthood (van Praag et al., 1999). However, most studies on exercise during adulthood have used animals that were singly housed in order to ensure sole access to running wheels by the rodents (Creer et al., 2010; Dostes et al., 2016; Kohman et al., 2012; Alexis M. Stranahan et al., 2007). It is possible that the housing conditions in the current study (pair housing) may have provided an enriched environment for adult rats resulting in elevated baseline levels of neurogenic cells which was not possible to enhance by exercise in adult rats but only by exercise during adolescence. Indeed, a recent report demonstrates that voluntary exercise by pair-housed mice during adolescence increased the survival of new neurons in the DG, an effect that was blunted in single-housed mice (Kozareva, O'leary, Cryan, & Nolan, 2018). However, it may also be the case that rodents that are pair-housed under control conditions (in the absence of exercise) during adolescence are also more vulnerable to their social surroundings, which is borne out in a lower level of neurogenesis that we observed compared to control animals. While this line of enquiry requires further investigation, these observations reinforce the notion that adolescence is a particularly sensitive time to environment

and sociability. Furthermore, single housing during early life has extensive deleterious effects on neurodevelopment (Fone & Porkess 2008; Morrissey, Mathews & McCormick 2011; Arakawa 2018). Therefore, pair housing was employed in the present study to avoid potential confounds associated with housing-induced stress effects on cognition and neurodevelopment. In the present study, exercised animals were compared to control animals in standard housing, and while we and others have observed exercise-induced increase in hippocampal-dependent behaviours using sedentary control rodents without a locked running wheel in the cage (Kozareva et al., 2018; O'Leary, Hoban, Cryan, O'Leary, & Nolan, 2018), future studies may include a secondary control group with a locked running wheel in order to correct for the novelty of a running wheel within the home-cage.

Notwithstanding that considerable plasticity and neurogenesis are maintained throughout the lifespan and into older age, the effects of exercise on cognitive function can change with age, possibly due to age-related changes in baseline levels of plasticity (Burke & Barnes, 2006; Duzel, van Praag, & Sendtner, 2016). Thus, exercise may have differing effects dependent upon the developmental period during which exercise occurs. The specific reasons for changes in plasticity with age may stem from metabolic or transcriptional changes associated with ageing, such as a decline in growth hormones like IGF-1 or BDNF (Barzilai, Huffman, Muzumdar, & Bartke, 2012; Kirk I Erickson et al., 2010) or with changes in angiogenesis. Indeed, earlier work has suggested that the neuronal capacity for plasticity may be constrained by the rate of vascularization (Wallace, Withers, Farnand, Lobingier, & McCleery, 2011). However, further work is needed to fully elucidate the role of these factors in exercise-induced changes in hippocampal plasticity and neurogenesis.

Aside from exercise during older age, exercise has been proposed as a tool to improve hippocampal plasticity and function across earlier age groups (Duzel et al., 2016; Kandola, Hendrikse, Lucassen, & Yucel, 2016; Voss et al., 2013). Studies conducted in humans have demonstrated that exercise during early life, such as during the pre-adolescent period (Chaddock et al., 2010; Chaddock, Pontifex, Hillman, & Kramer, 2011), adolescence (Herting & Nagel, 2012) and young adulthood (Stroth, Hille, Spitzer, & Reinhardt, 2009) improves learning and memory and is associated with larger hippocampal or temporal lobe volume (Chaddock et al., 2010). Similar findings have also been reported in adult cohorts, with exercise correlating with improvements in spatial memory and hippocampal volume (K. I. Erickson et al., 2011). Moreover, exercise during adulthood or middle-age has been shown to convey protective effects against the cognitive decline associated with ageing and neurodegenerative disorders (Duzel et al., 2016; Ryan & Nolan, 2016) as well as to offer stress resilience in rats (Kochi et al., 2017; Patki, Li, et al., 2014; Patki, Solanki, et al., 2014). Specifically, exercise during middle-age has been shown to improve memory function and enhance plasticity in aged mice (Marlatt, Potter, Lucassen, & van Praag, 2012). However, this protective effect has been suggested to be a function of the level of exercise undertaken (Gregoire et al., 2018; Naylor, Persson, Eriksson, Jonsdottir, & Thorlin, 2005) and it is not properly understood if exercise beginning early in life, such as preadolescence or adolescence, maintains the protective and pro-cognitive effects in old age after exercise has ended, or if the benefits of exercise are limited to a specific window when the physical activity occurs. Indeed, the study presented here demonstrated that exercise beginning in adolescence and maintained into early adulthood increases hippocampal plasticity to a greater degree than exercise beginning in adulthood. However, this finding could be

expanded to investigate if persistent or shorter periods of exercise beginning early in life provides benefits in older age or in terms of later disease vulnerability or resilience.

In conclusion, adolescent-initiated exercise resulted in a differential effect on hippocampal-dependent and independent cognition, neurogenesis and neurite arborisation in adulthood compared to exercise initiated during adulthood. Thus, environmental influences such as exercise during this critical period of brain development, may have long lasting effects on cognitive processes in later life.

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Figure legends:

Figure 1: Experimental timeline: Outline of the experimental timeline for rats undergoing exercise during adulthood (A) or adolescence (B). All rats were pair housed in standard housing (control) or with continuous access to a running wheel (exercise). Exercise began at either 8 or 4 weeks of age and continued throughout testing for 11 weeks for the adult-imitated exercise and 12 weeks for the adolescent-initiated exercise. Behavioural testing commenced after 4 weeks of exercise and tissue was collected for immunohistological analysis of neurogenesis and neurite complexity.

Figure 2 Running distance: Mean running wheel activity (km) per 24h for the duration of the study at as a result of adult and adolescent-initiated exercise. Data are graphed as means ± SEM (n=10).

Figure 3 Exercise during adolescence preferentially promotes performance in a location discrimination task: Representative image of large separation location discrimination screen (A). Trials to first reversal of the large location discrimination condition after exercise was initiated in adulthood (B) or during adolescence (C). Representative image of small separation location discrimination screen (D). Trials to first reversal of the small location discrimination condition after exercise was initiated in adulthood (E) or during adolescence (F). Data are graphed as means ± SEM (n=8-10).

Figure 4 Exercise during both adolescence and adulthood enhances location discrimination reversal learning: Representative image of large separation location discrimination screen (A).

Reversal learning in the large location discrimination condition after exercise was initiated in adulthood (B) or during adolescence (C). Representative image of small separation location discrimination screen (D). Reversal learning in the small location discrimination condition after exercise was initiated in adulthood (E) or during adolescence (F). (* $p<0.05$, ** $p<0.01$). Data are graphed as means ± SEM (n=8-10).

Figure 5 Exercise that began in adolescence impairs spontaneous alternation but has no effect on novel object recognition: Spontaneous alternation of rats after exercise was initiated in adulthood (A) or during adolescence (B). Novel object recognition of rats after exercise was initiated in adulthood (C) or during adolescence (D). (** $p<0.01$). Data are graphed as means +SEM (n=10).

Figure 6 Exercise that began in adolescence but not adulthood increased hippocampal neurogenesis: Mean number of DCX+ cells per section in the whole (A), dorsal (B) or ventral (C) hippocampus of rats after exercise was initiated in adulthood. Mean number of DCX+ cells per section in the whole (E), dorsal (F) or ventral (G) hippocampus of rats after exercise was initiated during adolescence. Representative images through the DG of DCX+ cells from adult sedentary control or exercised rats (D) and adolescent sedentary control or exercised rats (H). Scale bar = 50 μm. Higher magnification images depict immunopositive cells in the DG for DCX-positive cells. Scale bar = 10μm (* $p<0.05$). Data are graphed as means +SEM (n=3-5).

Figure 7 Adolescent and adult-initiated exercise increased neurite complexity of dorsal hippocampal DCX-positive cells: Neurite length of DCX+ cells in the dorsal and ventral DG

of rats after exercise was initiated in adulthood (A) or during adolescence (D). Number of neurite branch points on DCX+ cells in the DG of rats after exercise was initiated in adulthood (B) or during adolescence (E). Number of neurites per DCX+ cell of rats after exercise was initiated in adulthood (C) or during adolescence (F). Representative traces of DCX+ cells in the DG of adult (G) or adolescent (I) sedentary control rats, and in the DG of rats after exercise was initiated in adulthood (H) or during adolescence (J). Scale bar = 10 μm. (*$p<0.05$, **$p<0.01$). Data are graphed as means +SEM (n=4-5).

Figure 8 Adolescent-initiated exercise increased neurite complexity of dorsal hippocampal DCX-positive cells to a greater degree than adult-initiate exercise: Neurite length of DCX+ cells (A). Number of neurite branch points on DCX+ cells (B). Number of neurites per DCX+ cell (C). (*$p<0.05$, **$p<0.01$). Data are graphed as means +SEM (n=4-5).

Figure 9 Correlation of neurite length of DCX-positive cells in the dorsal hippocampus and cognitive flexibility: Correlation of reversal learning in the small separation condition and neurite length after exercise was initiated in adulthood (A) or adolescence (C). Correlation of reversal learning in the large separation condition and neurite length after exercise was initiated in adulthood (B) or adolescence (D). Data graphed as means (n=8).