The Long-Term Effects of Behavioural Interventions on Age-Associated Alterations in Hippocampal Neurogenesis and Memory Interference

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The process of normal aging results in changes in several cognitive abilities, including the ability to reduce interference between similar pieces of information upon recall. Aging has also been associated with changes in the hippocampus, including suppression of adult hippocampal neurogenesis, which may be associated with age-related changes in memory interference reduction. Behavioural interventions such as exercise and environmental enrichment, on the other hand, have been shown to stimulate adult hippocampal neurogenesis. The present study therefore used an animal model to assess the long-term effects of exercise and environmental enrichment on memory interference reduction and adult hippocampal neurogenesis in the aging dentate gyrus. Old rats were randomly assigned to standard cages, cages equipped with a running wheel, or enriched cages. Young rats were housed in standard cages and used as a control. All rats received BrdU injections at the start of the new housing conditions. Following nine months of the intervention, all rats were tested on a delayed nonmatching to place (DNMP) radial arm water maze task (RAWM) with a high interference component. On trials with the highest interference, old control animals performed significantly worse compared to young controls. No differences were found between young controls and old animals in the exercise or enrichment groups, suggesting that these behavioural interventions may have a protective effect against age-related deficits in interference reduction. Cellular analyses revealed significantly greater BrdU-labeled neurons at ten months post-injection in young controls compared to all aged animals, regardless of intervention. Results indicate that neurons generated in adulthood are capable of surviving for at least ten months, although aging significantly reduces survival regardless of behavioural intervention. Findings indicate that behavioural interventions implemented in middle age seem to be capable of preserving cognitive abilities later in life, although the mechanism by which this occurs remains to be further investigated.

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"Be transformed by the renewing of your mind." –Romans 12:2

Glory to God for all things.

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List of Abbreviations

Arc	Activity-regulated cytoskeleton-associated protein
BBB	Blood-brain barrier
BrdU	5-bromo-3'-deoxyuridine
CA	Cornu ammonis
CR	Calretinin
DCX	Doublecortin
DG	Dentate gyrus
DNMP	Delayed non-matching-to-place
EC	Entorhinal cortex
FGF-2	Fibroblast growth factor 2
GABA	Gamma-aminobutyric acid
GC	Granule cell
IEG	Immediate early gene
IGF-1	Insulin-like growth factor 1
LTP	Long-term potentiation
M.O.M.	Mouse-on-mouse
MAM	Methylazoxymethanol
MWM	Morris water maze
NeuN	Neuronal nuclei
NMDA	N-methyl-D-aspartate
OCT	Optimal cutting temperature
RAM	Radial arm maze

RAWM	Radial arm water maze
SSC	saline sodium citrate
TBS	Tris-buffered saline
TSA	Tyramide signal amplification
VEGF	Vascular endothelial growth factor

The Long-Term Effects of Behavioural Interventions on Age-Associated Alterations in Hippocampal Neurogenesis and Memory Interference

The process of aging is universal and indiscriminate, yet there is great variability in age-associated cognitive decline amongst individuals. While some individuals experience pathological conditions not typical of normal aging, such as Alzheimer's disease, others experience less severe cognitive changes associated with the normal aging process. The distinction between cognitive changes typical of normal aging and those associated with pathological conditions is one that must be made in order to distinguish between normal and pathological states. Moreover, it is important to investigate the biological mechanisms underlying cognitive changes during normal aging in order to understand the more extreme deficits observed with pathological aging, so that interventions may be developed (Erickson & Barnes, 2003).

Normal cognitive aging tends to include a decline in certain cognitive abilities, including conceptual reasoning, processing speed, and certain aspects of executive functioning (Harada, Natelson Love, & Triebel, 2013), in the absence of an age-related disease. The normal aging process is often accompanied by declines in both episodic and semantic memory, with the latter showing declines later in life compared to the former (Rönnlund, Nyberg, Bäckman, & Nilsson, 2005). Memory processes, such as acquisition, retention, and retrieval are also affected by normal aging, but to different extents. While retention of previously learned information is relatively spared (Whiting & Smith, 1997), acquisition (Haaland, Price, & Larue, 2003) and retrieval of newly learned information (Economou, 2009; Haaland et al., 2003; Price, Said, & Haaland, 2004) decline as a result of the normal aging process.

To study the normal aging process, rodent models have been used, particularly because rodents display individual differences in cognitive aging, much the same way that humans do, and do not experience neurodegenerative diseases, such as Alzheimer's disease (Gallagher & Rapp, 1997). One particular form of memory that is impacted by the aging process is spatial memory, as demonstrated by rodent models and the use of behavioural spatial tasks, which require a subject to learn and use information about the spatial properties of the environment (Rosenzweig & Barnes, 2003). Aging has been shown to result in deficits in performance on several spatial learning tasks, including the Barnes circular platform, the radial arm maze, and the Morris water maze (for review, see Rosenzweig & Barnes, 2003). Spatial learning and memory in rodents require both allocentric (involving the use of distal cues/cues outside the organism) and egocentric (involving internal and proximal cues/self-movement) navigation. The former involves several brain regions, including the hippocampus and the entorhinal cortex (EC), which are involved in encoding allocentric, semantic, and episodic memory in humans (Vorhees & Williams, 2014). Interestingly, age-associated spatial memory decline largely mirrors the deficits observed in subjects with hippocampal damage, including rats (Jarrard, 1993), monkeys (Murray, Baxter, & Gaffan, 1998), and humans (Maguire, Burke, Phillips, & Staunton, 1996). While aging results in changes in multiple brain regions, the hippocampus has been shown to be preferentially targeted, undergoing both structural and physiological changes with increasing age (for review, see Rosenzweig & Barnes, 2003). The hippocampus is therefore a critical region to investigate when studying the effects of normal cognitive aging on spatial learning and memory.

The Hippocampus

The hippocampus proper, located in the medial temporal lobe, is one of several brain regions that form the hippocampal formation. The hippocampus proper can be subdivided into

the three cornu ammonis (CA) pyramidal cell layers: CA1, CA2, and CA3. The hippocampus proper, along with the dentate gyrus (DG), subiculum, presubiculum, parasubiculum, and EC form the hippocampal formation (Amaral & Witter, 1989; Witter, 2007).

The hippocampus has long been known to be a key structure involved in learning and memory (Marr, Willshaw, & McNaughton, 1991; Scoville & Milner, 1957). Both episodic and semantic memory – the conscious memory of specific autobiographical events and memory of facts about the world in general, respectively – are functions that have been attributed to the hippocampus (Friedman, Klivington, & Peterson, 2013). Lesion studies, however, have shown that the hippocampus exhibits functional subdivisions along the septotemporal axis. While the dorsal (posterior) hippocampus is preferentially involved in learning and memory, the ventral (anterior) hippocampus may be more involved in emotional processing associated with anxiety-related behaviours, in contrast to the amygdala, which is specifically associated with fear (Bannerman et al., 2004).

The hippocampus has been thought to create context-specific representations of individual events, which are necessary for recall, by integrating external sensory information and self-motion-based spatial information from the lateral and medial entorhinal cortices, respectively (Knierim, Lee, & Hargreaves, 2006). The EC is generally thought to be the starting point of the tri-synaptic circuit – a series of three sequential glutamatergic synapses through which information is processed in the hippocampus. Information from various neural networks, including the visual, auditory, and somatic associative cortices, is integrated as it passes through the parahippocampal and perirhinal cortices, then to the EC where it is then projected to and processed within the hippocampus (Lavenex & Amaral, 2000). Information passes mainly from layer two of the EC to the DG of the hippocampus via the perforant path axons. The granule cells

(GCs) located in the DG have mossy fibre axons – distinctive unmyelinated axons that project from the basal portion of GCs through the hilus to the proximal apical dendrites of the pyramidal cells in the CA3 region (Blaabjerg & Zimmer, 2007). Multiple synapses are formed between the mossy fibre axons and specialized spine clusters, such as thorny excrescences, on the dendrites of the CA3 pyramidal cells (Blaabjerg & Zimmer, 2007). From the CA3, Schaffer collateral axons project to CA1 pyramidal cells. CA3 neurons that are more distant from the CA1 project mainly to the apical dendrites of CA1 pyramidal cells, while those located more closely project primarily to the basal dendrites (for review, see Spruston, 2008).

In addition to the tri-synaptic circuit, processing of spatial information occurs along alternate pathways, such as the direct projections from layer 2 of the EC to the CA3 and from layer 3 to the CA1 (McNaughton, Barnes, Meltzer, & Sutherland, 1989). CA3 axons also project through commissural fibres to the dendrites of contralateral CA3 cells, as well as forming a recursive feedback loop by projecting to the dendrites of the CA3 cells from which they originated (Blaabjerg & Zimmer, 2007). The presence of the CA3 recurrent collaterals has led to the suggestion that the CA3 may function as an auto-associative network involved in both memory storage and recall (Blaabjerg & Zimmer, 2007).

The role of the hippocampus in the formation of spatial memories has been consistently demonstrated through a variety of experiments using electrophysiological, lesion, genetic, and neuroimaging approaches (Burgess, Maguire, & O'Keefe, 2002; S. Leutgeb, Leutgeb, Moser, & Moser, 2005; Morris et al., 2003; Nakazawa, McHugh, Wilson, & Tonegawa, 2004; O'Keefe & Nadel, 1978). Hippocampal subregions have been shown to be involved in different aspects of spatial learning and memory. Genetically modified mice lacking the gene encoding N-methyl-D-aspartate (NMDA) receptors, which are involved in synaptic plasticity and memory, specifically

in CA1 pyramidal cells, have been found to show specific impairments in spatial memory in the Morris water maze (MWM) task, but not in the landmark task – a water maze task designed to assess non-spatial learning (Tsien, Huerta, & Tonegawa, 1996). Furthermore, while these genetically modified mice retain place-related activity within the CA1, there is a reduction in spatial specificity of place fields, resulting in a disruption of coordinated firing between pairs of neurons even if they possess overlapping fields (McHugh, Blum, Tsien, Tonegawa, & Wilson, 1996). Such findings demonstrate the importance of the CA1 in spatial memory abilities. The CA1 region has also been shown to be specifically involved in long-term spatial memories. Lesions to the direct EC projection to the CA1 did not affect short term (24 hours) spatial memory of the MWM, but resulted in specific deficits in long term (four weeks) spatial memory (Remondes & Schuman, 2004). By subjecting trained animals to lesions following either a 24 hour delay or a three week delay, long term memory deficits were suggested to be a result of a disruption of consolidation of memories, rather than acquisition (Remondes & Schuman, 2004). The CA1 region of the hippocampus has therefore been shown to be important for spatial memory, specifically the consolidation of long-term spatial memories.

In addition to the CA1, the DG has been shown to play an essential role in memory formation (Ming & Song, 2005; Zhao, Deng, & Gage, 2008). Specifically, destroying over 75% of the GCs of the DG in both hemispheres using the neurotoxin colchicine has been shown to result in severe deficits in spatial learning abilities in freely moving rats (McNaughton et al., 1989). On the other hand, the CA3 has been suggested to be responsible for associative learning, contributing to episodic memory. Specifically, blocking CA3 NMDA receptors or lesioning the CA3 was found to result in impaired performance on a delayed non-matching-to-place (DNMP) radial arm maze (RAM) task, but only when the task was conducted in a novel environment, not

in a familiar environment (Lee & Kesner, 2002, 2003). The CA3 has been suggested to play a role in learning new spatial environments, but not necessarily in finding familiar locations (Nakazawa et al., 2003). Importantly, the DG as well as the CA3 have been suggested to perform the specific functions of pattern separation and pattern completion, respectively.

Pattern separation and pattern completion. Pattern separation refers to the ability to reduce the overlap between similar incoming pieces of information by mapping overlapping patterns of neural activation to less overlapping representations. By storing similar pieces of information in distinct orthogonalized representations, pattern separation is one mechanism that reduces memory interference upon recall (Becker, 2017; Yassa & Stark, 2011). The term pattern separation is often used to refer to two distinct processes: neural pattern separation and behavioural pattern separation. However, defining pattern separation as a behaviour is problematic given that pattern separation refers to the distinct neuronal activities of a brain circuit for similar stimuli (Leutgeb, Leutgeb, Moser, & Moser, 2007). The behaviour, on the other hand, refers to the act of reducing interference in order to behaviourally discriminate between two similar items (Clelland et al., 2009).

The DG is thought to engage in pattern separation on the input received from the EC, sending orthogonalized representations to the CA3 via the mossy fibres (Treves & Rolls, 1994). To allow the discrimination of similar inputs to occur, different activity patterns are generated in one of two ways: distinct sets of neurons are activated or the firing rates in the same set of neurons are modulated (Deng, Aimone, & Gage, 2010; Deng, Mayford, & Gage, 2013; S. Leutgeb, Leutgeb, Treves, Moser, & Moser, 2004).

A related but distinct process known as pattern completion refers to the use of previously stored representations to form complete representations from partial or degraded input patterns,

providing accurate generalizations when only partial sensory input is available (Yassa & Stark, 2011). Marr had originally suggested that recurrent collaterals allowed brain regions to function as auto-association networks involved in pattern completion (Marr et al., 1991). Given the presence of recurrent collaterals in the CA3, many have hypothesized that the CA3 functions as a pattern completion network (Rolls, 1996; Treves & Rolls, 1994).

Pattern-separated representations have been thought to be forced onto CA3 neurons from the mossy fibre pathway linking the DG and the CA3 in order to reduce interference. Interestingly, the EC has been shown to have direct projections reaching the CA3, which, although weaker, have been suggested to provide cues for recall (Rolls, 2007). Lesioning the perforant path and mossy fibres eliminates the major afferent inputs to the CA3 and results in deficits in performance on the modified Hebb-Williams maze, a simple navigation test (Lee & Kesner, 2004b). Specifically, rats that underwent electrolytic lesioning of the perforant path from the EC exhibited deficits during retrieval as indicated by the lack of a reduction in the number of errors made between days. In contrast, DG-lesioned rats displayed deficits in encoding, as indicated by a lack of change in the number of errors within a day (Lee & Kesner, 2004b). The differential effect of lesioning the afferent inputs to the CA3 has led to the hypothesis that the CA3 works to integrate input from both the mossy fibres from the DG and the perforant path from the EC to assist in recall (Yassa & Stark, 2011).

Experimental work has further demonstrated the critical role of the DG in performing memory tasks that have a high interference component. Genetically modified mice lacking NMDA receptors that were tested on a fear-conditioning task showed specific deficits when they had to differentiate between two similar contexts (McHugh et al., 2007). In addition, context-modulated place cell activity was reduced in CA3 pyramidal cells when mice had to distinguish

two similar contexts. The authors concluded that NMDA receptors present in the synaptic connection between the perforant path axons and the GCs of the DG are important for an animal's behavioural discrimination abilities (McHugh et al., 2007). Another study using genetically modified mice lacking NMDA receptors also found significant impairment in long-term potentiation in perforant path input to the DG, as well as impairment in spatial working memory on a RAM task (Niewoehner et al., 2007).

Dentate GCs have been thought to be ideal for pattern separation, given that they are more numerous than cells in other hippocampal regions (Amaral, Ishizuka, & Claiborne, 1990), they fire sparsely (Danielson et al., 2016; J. K. Leutgeb et al., 2007), and they form strong synapses with CA3 pyramidal cells (Rollenhagen et al., 2007). These features thus make non-overlapping representations easier to create (Gibson, Robinson, & Bennett, 1991), suggesting that DG GCs are responsible for orthogonalizing input received from the EC and sending it to the CA3 (Chavlis & Poirazi, 2017). In addition to the aforementioned characteristics, the DG is also capable of neurogenesis throughout life. New neurons generated in adulthood in the DG have specifically been shown to play an important role in both spatial memory and overcoming memory interference.

Adult Hippocampal Neurogenesis

The subgranular zone of the DG is unique in that it is one of two mammalian brain regions, along with the subventricular zone of the lateral ventricles, where neurogenesis – the generation of functionally integrated neurons from progenitor cells – continues postnatally and throughout adulthood. Initial reports of neurogenesis in the adult mammalian brain identified new neurons in the DG (Altman & Das, 1965), the neocortex (Altman, 1966), and the olfactory bulb (Altman, 1969) in adult rats. The use of 5-bromo-3'-deoxyuridine (BrdU), a thymidine

analog that is incorporated into DNA during the S phase of the cell cycle, has been used to identify newly born cells (Cooper-Kuhn & Kuhn, 2002; Eriksson et al., 1998). BrdU can be incorporated into any type of replicating cell, including glial cells such as astrocytes; therefore, BrdU is often used in combination with cell-specific markers, such as neuronal nuclei (NeuN) which is expressed by mature neurons, to identify newly differentiated neurons using immunohistochemical techniques (Mullen, Buck, & Smith, 1992). Such techniques have provided evidence for the occurrence of neurogenesis in adulthood in several species (Gould, Tanapat, McEwen, Flügge, & Fuchs, 1998; Guéneau, Privat, Drouet, & Court, 1982; Kuhn, Dickinson-Anson, & Gage, 1996), as well as in the human hippocampus (Eriksson et al., 1998).

Neurogenesis occurs in three sequential stages: proliferation, maturation, and differentiation. Beginning in the subgranular zone of the DG, radial and non-radial neural precursors proliferate and give rise to intermediate progenitors, which then become neuroblasts. These immature neurons differentiate into GCs with the morphological and phenotypic characteristics of neurons (Eriksson et al., 1998). In juvenile rats (38 days old), dividing progenitor cells can produce approximately 4000 new neurons per day (McDonald & Wojtowicz, 2005). Upon migrating to the inner GC layer of the DG (Ming & Song, 2005), newly generated GCs form dendritic processes, which extend to the molecular layer, and axon fibres, which project to the CA3 through the hilus (Zhao, Teng, Summers, Ming, & Gage, 2006). The interaction between new neurons and the interneurons and mossy cells of the hilus, as well as the CA3 pyramidal cells, allows the formation of synaptic connections, which may promote cell survival (Zhao et al., 2006).

Interestingly, not all immature neurons, which express markers such as doublecortin (DCX), fully develop into mature GCs (Kempermann, Gast, Kronenberg, Yamaguchi, & Gage,

2003). Neurogenic brain areas have been shown to contain a significantly greater number of apoptotic cells than non-neurogenic areas, such as the cerebellum, contributing to a sharp non-linear decline in the number of new cells (Biebl, Cooper, Winkler, & Kuhn, 2000). In fact, approximately 20% of the BrdU-labeled cells that were present following 1 day of BrdU injection were present at four weeks post-injection (Kempermann et al., 2003). However, cells that have been found to survive to the four-week mark appear to persist for several months, with no significant difference in the number of BrdU-labeled cells at four weeks compared to 11 months post-injection (Kempermann et al., 2003). Several reports have found that neurons generated in adulthood that survive to maturity are stable and express mature neuronal markers (Biebl et al., 2000; Dayer, Ford, Cleaver, Yassaee, & Cameron, 2003; Kempermann et al., 2003), although this has been found to occur 1-2 weeks earlier in rats than in mice (Snyder et al., 2009). Despite differences in timing, adult-generated neurons in both rats and mice have been found to be functional as they are integrated into the hippocampal circuitry (van Praag et al., 2002). While new neurons in the DG of both adult rats and mice are incorporated into the hippocampal circuitry, they are ten times more likely to be incorporated in learning circuits in rats than in mice (Snyder et al., 2009). Upon reaching 6-8 weeks of age, adult-generated GCs are twice as likely to be integrated into spatial memory networks than existing mature GCs and become increasingly preferred as they mature, suggesting that neurons generated in adulthood may play a role in memory processing in the DG (Kee, Teixeira, Wang, & Frankland, 2007; but also see Stone et al., 2011). New neurons also differ from existing neurons in that they exhibit enhanced excitability and increased conductance, as well as a lower threshold for induction of long-term potentiation (LTP), compared to their mature counterparts under identical conditions (Ambrogini et al., 2004; Schmidt-Hieber, Jonas, & Bischofberger, 2004). Such characteristics have been

suggested to facilitate synaptic plasticity, which in turn may assist in the formation of new memories (Schmidt-Hieber et al., 2004).

Adult-generated neurons have also been shown to express both c-fos (Jessberger & Kempermann, 2003; Kee et al., 2007) and activity-regulated cytoskeleton-associated protein (Arc) (Ramirez-Amaya, Marrone, Gage, Worley, & Barnes, 2006) following hippocampal stimulation via participation in hippocampus-dependent tasks. In fact, compared to existing GCs, five-month-old adult-born GCs seem to have a lower threshold for Arc expression, with 2.8% more newborn cells expressing Arc than existing cells following a spatial exploration task (Ramirez-Amaya et al., 2006). Expression of immediate early genes (IEGs) not only indicates that new neurons are functional, but that they respond specifically to behavioural exploration, suggesting a role in spatial abilities.

Although the specific function of adult-generated neurons remains largely unknown, given that the DG is involved in learning and memory processes, and that the GC layer of the DG is where new neurons are added in adulthood, it has been suggested that these new neurons play a role in learning and memory (Gould, Tanapat, Hastings, & Shors, 1999; Kempermann, 2002; Schinder & Gage, 2004), as well as in the encoding of contextual information (Saxe et al., 2006; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002). Since the DG is the only region of the hippocampus where neurogenesis occurs, it follows that newly generated neurons may play a role in the storage of distinct representations of novel events, but not in the retrieval of previously stored memories (Becker, 2005). In more recent years, a number of specific roles for adult born hippocampal neurons have been suggested, including a particular role in interference reduction.

Hippocampal neurogenesis and high interference memory tasks. Several studies have implicated adult-born neurons in high interference memory tasks. Clelland et al. (2009) used a DNMP RAM task to assess the performance of adult mice on a high interference memory task, following focal ablation of hippocampal neurogenesis. Mice had to differentiate between two arm locations and were expected to select the arm that had not previously been presented during the sample phase. Distance between the two arms varied – arms that were presented in close spatial proximity are thought to require a greater reduction of interference than arms that are farther apart (Clelland et al., 2009). Results showed that mice lacking hippocampal neurogenesis exhibited selective impairment on the task at low spatial separations (i.e., high interference conditions), but not at high separations (i.e., low interference conditions). Neurons generated in adulthood therefore seem to play a crucial role in reducing memory interference, particularly when an animal is required to discriminate between similar locations presented closely in space (Clelland et al., 2009).

The same group of researchers also explored the specificity of the deficits observed as a result of ablation of adult hippocampal neurogenesis – namely whether deficits could be seen in global hippocampal functioning or specifically in discrimination abilities. Mice were tested on a paired associates learning object-in-place task, which tests hippocampus-dependent spatial learning, as well as a two-choice spatial discrimination task using a touch screen to further assess behavioural discrimination. Irradiated mice showed no significant differences in performance on the spatial task compared to sham mice, suggesting that a complex hippocampus-dependent spatial task can be acquired at a normal rate without neurogenesis (Clelland et al., 2009). However, irradiated mice performed significantly worse on the two-choice spatial task compared to sham controls, indicating significant impairment in spatial discrimination abilities as a result

of the absence of neurogenesis (Clelland et al., 2009). Results of this study demonstrate the role of adult-born neurons specifically in high interference memory tasks, suggesting a possible role in memory interference reduction.

Adult-born neurons implicated in high interference memory tasks have been suggested to play a role in contextual discrimination, which allows animals to recognize familiar environments, identify changes to the environment, and discriminate between similar contexts (Lee & Kesner, 2004a). Using a transgenic model to selectively destroy neural precursors in the hippocampus, adult mice underwent a fear-conditioning task in two similar yet distinct contexts and were tested on their ability to differentiate between the contexts (Tronel et al., 2012). Mice were given an initial training phase, during which all mice were shown to be capable of contextual conditioning, suggesting, as Dupret et al. (2008) did, that neurogenesis does not play a role in this particular form of learning. During eight days of testing, however, the performance of control mice improved, while the transgenic mice showed no improvement in discrimination. In fact, transgenic mice showed deficits in their ability to differentiate between the two contexts following training (Tronel et al., 2012). Such findings not only suggest that training enhances contextual discrimination, but that adult-born hippocampal neurons may play a necessary role in this process, working to transform similar memories into non-overlapping representations. Conversely, augmentation of neurogenesis by genetically modifying mice to lack *Bax*, a pro-apoptotic gene, was found to significantly improve performance when mice were required to discriminate two similar contexts (Sahay et al., 2011). Increasing the survival of hippocampal GCs generated in adulthood therefore seems to elicit improvements in discrimination between similar contexts, further suggesting that adult-born neurons play a role in reducing memory interference.

Factors modulating adult hippocampal neurogenesis. Various factors have been shown to influence adult hippocampal neurogenesis. Aging in particular has consistently been shown to be associated with suppressed neurogenesis (McDonald & Wojtowicz, 2005), while environmental factors such as voluntary exercise and exposure to a cognitively stimulating environment have been suggested to stimulate neurogenesis in the hippocampus (J. Brown et al., 2003).

Aging. Adult-born GCs continue to be added to the DG throughout adulthood, including later life and senescence, in rodents (Altman & Das, 1965), non-human primates (Gould, Reeves, et al., 1999), and humans (Eriksson et al., 1998). GC density in the DG has been shown to increase in adulthood (Bayer, 1982) and remain at least constant during aging (Bondareff & Geinisman, 1976); however, the production of new GCs drastically declines with age (Cameron & McKay, 1999; Heine, Maslam, Joëls, & Lucassen, 2004; Kuhn et al., 1996; Nacher, Alonso-Llosa, Rosell, & McEwen, 2003; Seki & Arai, 1995), suggesting that the aging process may be associated with a reduction in the production of new cells, rather than a loss of existing neurons. Specifically, rats have been found to experience a 94% decline in net neurogenesis and a 92% reduction in GC production during the first year of life (McDonald & Wojtowicz, 2005). While dividing progenitor cells in the DG of 38 day old rats can produce up to 4000 new neurons each day, those in middle aged (12 months old) rats experience a drastic reduction, producing only approximately 250 per day (McDonald & Wojtowicz, 2005). Furthermore, the density of BrdU-labeled cells in the DG of aged rats (21 months old) 4-6 weeks post-injection was found to be only 10% of the density measured in 6-month-old rats (Kuhn et al., 1996). Similarly, the density of cells labeled with polysialylated-neural cell adhesion molecule (PSA-NCAM) – a marker for migration and differentiation involved in the maturation of GCs – also decreased significantly in

aged rats to 12% of the density in young animals (Kuhn et al., 1996). The specific reduction in cell production with age has prompted researchers to suggest that the age-associated decline in neurogenesis is, at least in part, a result of changes to the proliferative activity of the hippocampal progenitor cell population (McDonald & Wojtowicz, 2005).

Age-related suppression of neurogenesis has been suggested to play a role in the cognitive decline typically seen with increasing age. A significant negative correlation has been found between the number of adult-generated GCs in the DG of aged (twenty months old) rats, but not young (three months old) rats, in the distance swam and latency to find the hidden platform in the Morris water maze (Drapeau et al., 2003). In addition, the number of surviving adult-born neurons has been shown to positively correlate with memory performance, such that animals that performed the best on the water maze task also had the highest number of adult-born cells that survived at least three weeks following their birth, suggesting that spatial memory abilities tend to be preserved with age in individuals with a higher level of hippocampal cell proliferation (Drapeau et al., 2003). Cell differentiation has also been found to be associated with performance, with aged rats with preserved performance on the learning task having more adult-born cells with a neuronal phenotype compared to aged rats that exhibited impairments (Drapeau et al., 2003). Conversely, other studies have found no relation between cognitive decline and reductions in GC production over the lifespan, despite a 40% reduction in GC proliferation occurring by middle age (13 months old) and an 85-90% reduction occurring in old age (25 months old) compared to the levels found in young 7-month-old rats (Bizon & Gallagher, 2003; Merrill, Karim, Darraq, Chiba, & Tuszynski, 2003). However, discrepancies in the findings of these studies may be attributed to methodological differences, such as differing BrdU injection protocols and the use of different strains and sex of rats. Such methodological differences have

been shown to influence neurogenesis (Banasr, Hery, Brezun, & Daszuta, 2001; Galea & McEwen, 1999; Kempermann, Kuhn, & Gage, 1997; Perfilieva, Risedal, Nyberg, Johansson, & Eriksson, 2001; Tanapat, Hastings, Reeves, & Gould, 1999) and may have contributed to the discordance of the findings. Thus, the association between age-related suppression of neurogenesis and aged related spatial memory decline is still not fully understood.

Remarkably, while GC production declines with age, differentiation and maturation of the remaining newborn cells do, in fact, proceed normally (McDonald & Wojtowicz, 2005; Rao, Hattiangady, Abdel-Rahman, Stanley, & Shetty, 2005). Adult-born GCs in the DG of middle-aged and aged rats experience a delay in migration to the GC layer, as well as slower maturation, compared to young rats. However, this initial delay ultimately has no effect since, at five months old, the percentage of new cells that have migrated to different areas of the GC layer have not been found to significantly differ between the three age groups (Rao et al., 2005). Furthermore, despite a delay in the expression of NeuN, adult-born GCs in middle-aged and aged rats were still shown to be capable of expressing this marker of neuronal maturation (Rao et al., 2005). McDonald and Wojtowicz (2005) similarly found that hippocampal neurons generated in aged rats are capable of differentiating, maturing, and migrating to the GC layer, in a manner similar to that of their juvenile counterparts.

Not only do hippocampal neurons generated in old age follow the same developmental trajectory as those generated in younger individuals, these neurons have also been shown to be functional. Adult-generated neurons in both young and old animals express Arc in response to spatial exploration during the Morris water maze task in a significantly greater proportion than the remaining cells of the DG (Marrone, Ramirez-Amaya, & Barnes, 2012). Furthermore, adult-born neurons that are generated in both young and aged animals have been shown to be equally

likely to express Arc in response to spatial exploration (Marrone et al., 2012). Taken together, these studies show that aging significantly reduces cell proliferation, but that differentiation, maturation, and migration proceed normally. In addition, GCs generated in senescence are capable of becoming functionally integrated in the hippocampal circuitry. However, the relation between age-related suppression of adult hippocampal neurogenesis and age-related cognitive decline is not as conclusive. Additionally, declining rates of neurogenesis with increasing age may play a role in age-related deficits in memory tasks with a high level of interference. In fact, a number of human studies support a link between age-related suppressed hippocampal neurogenesis and age-related deficits in reducing memory interference.

Healthy young (mean age of 23 years) and older adults (mean age of 75 years) were tested on a continuous recognition task in which they were shown pictures of a series of objects and then required to identify objects as either novel, repeated, or similar (known as "lures"). The ability to correctly identify lures as "similar" rather than "old" was used as the memory interference task. Older adults exhibited behavioural impairment in reducing interference compared to young controls. Specifically, older adults experienced greater difficulty when lures were more similar – that is to say, they required a greater degree of dissimilarity between objects in order to be able to correctly identify them (Yassa et al., 2011). Deficits in reducing memory interference may also be associated with age-related suppression of adult hippocampal neurogenesis. Given that adult-born neurons have been shown to be involved in reducing memory interference (Clelland et al., 2009), declining rates of neurogenesis observed with aging may underlie age-related deficits in task with high level of memory interference. Taken together, the data suggests that restoring cell proliferation to the level of younger animals may potentially reverse, or alleviate, age-associated deficits in memory interference reduction.

Exercise and environmental enrichment. Voluntary exercise and environmental enrichment have been shown to have a positive effect on adult hippocampal neurogenesis. Exercise has been shown to induce a significant positive effect on cell proliferation, survival, and differentiation in the DG. Mice that were housed in standard cages with the addition of a running wheel had significantly higher rates of proliferation compared to mice that were in other conditions, including water-maze learning, swim-time-yoked controls, enriched environment, and standard housing (control) groups (van Praag, Kempermann, & Gage, 1999). Exercise has also been shown to have a positive effect on survival of newborn neurons by increasing cell survival to 201% of the level of controls (van Praag et al., 1999).

Similarly to exercise, environmental enrichment has been shown to positively affect cell differentiation, with a significantly greater number of surviving GCs expressing NeuN in mice exposed to environmental enrichment compared to controls and swimmers (van Praag et al., 1999). While environmental enrichment was not shown to have an effect on cell proliferation in this study, a study by Kempermann using C57BL/6 mice found that environmental enrichment resulted in twice as many proliferating cells in the DG compared to those exposed to standard housing, suggesting that genetics may play a role in the effect of environmental enrichment on the different phases of neurogenesis (Kempermann, Brandon, Gage, & Gage, 1998). Environmental enrichment seems to also have a specific effect on the survival of adult-born neurons. While the number of BrdU-labeled cells in the DG of enriched rats was not found to significantly differ from that of rats housed in standard cages at one day post-BrdU injection, there was a significant difference in the number of BrdU-labeled cells at four weeks post-injection (Nilsson, Perfilieva, Johansson, Orwar, & Eriksson, 1999). Environmental enrichment therefore does not seem to have a particular effect on cell proliferation, but rather promotes

survival of adult-born hippocampal cells arising from progenitor cells in the subgranular layer (Nilsson et al., 1999). Other studies have similarly found a significant increase in the number of surviving neurons in enriched mice compared to controls, with increases in labeled cells ranging from 67% (Kempermann et al., 1998) to 175% (van Praag et al., 1999) at four weeks post-injection.

The upregulation of hippocampal neurogenesis in response to both exercise and environmental enrichment has been shown to persist in later adulthood. Interestingly, the proliferative effect of voluntary wheel running was shown to weaken with continued exercise; however, continued exercise in old age decreases the age-related suppression of neurogenesis, with progenitor cells remaining capable of activity-induced proliferation at both one (middle age) and two (old age) years of age (Kronenberg et al., 2006). In old animals, exercise increased progenitor cell division to a level comparable to that of their juvenile counterparts. Similarly, the total number of BrdU-labeled cells at four weeks post-injection was found to be significantly reduced in old controls (18 months old) compared to young controls (6 months old), but increased by 68% and 32% in young and old mice, respectively, when exposed to enrichment (Kempermann, Kuhn, & Gage, 1998). A significantly greater proportion of BrdU-labelled cells were also NeuN-positive in both young and old enriched mice compared to their standard-housed counterparts. However, of note, the survival of adult-born GCs was not assessed past four weeks post-injection. Regardless, physical activity and environmental enrichment are potential methods of increasing neural plasticity in old age through their effects on proliferation and survival of adult-generated neurons.

Increasing neurogenesis through exercise and environmental enrichment may help reduce age-related cognitive deficits. Spatial learning abilities are significantly improved through both

physical activity and enrichment. Using a standard radial arm maze task, rats that were provided with voluntary access to a running wheel required 30% fewer trials in order to reach the daily criterion for maze completion compared to controls, despite spending the same average amount of time in each arm (Anderson et al., 2000). Survival of adult-born neurons in response to environmental enrichment also seems to be correlated with enhanced spatial learning abilities, as measured by the MWM task, in both young (Nilsson et al., 1999; Segovia, Yagüe, García-Verdugo, & Mora, 2006) and old animals (Kempermann et al., 1998b). In addition, the effect of environmental enrichment on spatial learning has been shown to persist, rather than wear off, with prolonged exposure. Mice that were exposed to environmental enrichment for eleven months exhibited a sustained effect on performance in the MWM task, with enriched mice exhibiting a learning curve significantly below that of the control group, as well as behavioural changes such as greater locomotor skills, greater endurance, and more adaptive exploration compared to controls (Kempermann, Gast, & Gage, 2002). Such behavioural changes mirrored the survival-promoting effect of enrichment on adult-born neurons, such that mice housed in an enriched environment possessed a significantly greater number of BrdU/NeuN double-labeled cells (26% in enriched mice compared to 8% in control mice) (Kempermann et al., 2002). However, similar to previously mentioned studies (Kempermann et al., 1998b), mice were injected with BrdU at 21 months of age and survival of BrdU-labeled cells was assessed at 28 days post-injection. Therefore, the effect of prolonged exposure to environmental enrichment on the long-term survival of new neurons is still unknown.

Importantly, the enrichment paradigms that have been discussed thus far have included an exercise component as part of the enriched environment; however, studies that have used a model of environmental enrichment that does not include a running wheel have still found an

effect on neurogenesis (Bruel-Jungerman, Laroche, & Rampon, 2005) and on long-term memory retention measured by the novel object recognition task, with both 24 and 48 hour delay intervals between the two phases of the task (Bruel-Jungerman et al., 2005). In addition, when methylazoxymethanol (MAM) – an antimitotic agent – was used to suppress hippocampal neurogenesis, long-term memory retention in enriched rats was impaired, such that enriched rats performed similarly to their standard-housed counterparts that had not received MAM treatment (Bruel-Jungerman et al., 2005). Therefore, environmental enrichment, in the absence of an exercise component, seems to have a specific survival-promoting effect on adult hippocampal neurogenesis, which in turn seems to play a role in memory retention. Exercise and environmental enrichment have thus been suggested to stimulate adult hippocampal neurogenesis, albeit in differing ways.

Rationale and Hypotheses

Based on the aforementioned studies, aging is associated with impaired performance in high interference memory tasks, which have been shown to relate to adult hippocampal neurogenesis (Clelland et al., 2009). Aging is also associated with suppressed adult hippocampal neurogenesis (Cameron & McKay, 1999; H. Y. McDonald & Wojtowicz, 2005). As summarized, interventions such as exercise (Creer, Romberg, Saksida, van Praag, & Bussey, 2010) and environmental enrichment (Kempermann et al., 1998b) have been shown to counteract the age-related suppression of hippocampal neurogenesis. Enhancing adult hippocampal neurogenesis through exposure to an enriched environment or exercise should therefore improve performance in high interference memory tasks. Thus, the first aim of the present study was to assess whether adult hippocampal neurogenesis and the ability to overcome memory interference are negatively affected by the normal aging process. The second aim of the study was to investigate whether

prolonged exposure to an enriched environment or voluntary exercise can enhance performance on a high interference memory task. We hypothesized that these two interventions would be capable of increasing performance of aged rats to a level comparable to that of their young standard-housed counterparts. Additionally, to our knowledge, the long-term survival of BrdU-labeled neurons has not been investigated under the prolonged influence of these pro-neurogenic interventions. In prior studies, the long-term effects of exercise and environmental enrichment have been studied on a cohort of new neurons usually labeled between 21 and 28 days prior to sacrifice (Creer et al., 2010; Kempermann et al., 2002). Thus, the third aim of the present study was to investigate whether prolonged exposure to environmental enrichment and voluntary exercise has any effect on the long-term survival of a cohort of neurons labeled at the beginning of the interventions. We hypothesized that the survival of labeled neurons in rats exposed to exercise or environmental enrichment would be comparable to that of young rats housed in standard conditions.

Methods

Animals

At the start of the experiment, thirty-five male Sprague Dawley rats (Harlan, USA) were used. Nine of the rats were two months old and the remaining twenty-six rats were eight-month-old retired breeders. All of the rats were single-housed in individual standard cages upon arrival prior to the start of the study. All rats followed a 12:12 h light/dark cycle (lights on at 8:00 am) with food and water available *ad libitum*. All procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the University of Waterloo's Animal Care Committee.

Drug Administration

Rats were administered intraperitoneal injections of 50 mg/kg of 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) dissolved in 37°C 0.9% physiological saline. Injections were delivered in a volume of 2 ml/kg body weight once a day for a total of six days.

Housing

Following the first injection, young rats were returned to their original standard cages and remained housed individually as before. Old rats were randomized into one of three housing conditions: standard individual cages as before, cages with access to a running wheel, and larger cages with two other rats and access to a changing set of toys. The BrdU injection continued for an additional five days in the new housing conditions. Rats remained in their respective housing conditions for nine months. Over the course of the intervention, three rats were euthanized due to age-related diseases, which disqualified them from the study. The remaining 32 rats therefore consisted of nine young controls, nine in the enriched environment, seven in the wheel cages, and seven old controls. During the intervention, all rats underwent the MWM task, a novel object recognition task, a location discrimination task, and lastly the radial arm water maze (RAWM) task prior to sacrifice (Figure 1). The current thesis is only related to the last behavioural task, the radial arm water maze.

Radial Arm Water Maze (RAWM)

The radial arm maze (RAM) consists of a central circular platform with arms extending from the centre that can be either opened or closed to allow or prevent the animal from entering. The RAM, similar to other maze tasks, has traditionally been used to assess place learning by testing an animal's ability to learn the location(s) of a food reward using visuospatial cues. Specifically, the RAM has been suggested to measure stable reference-memory performance and

has the potential to measure working memory as well (Hodges, 1996).

Clelland et al. (2009) adapted the traditional DNMP eight-arm RAM procedure in order to increase interference to assess spatial discrimination abilities (described in detail below). Typically, the RAM is conducted on land and uses food as a motivator. However, such a paradigm requires animals to be food deprived in order to increase task motivation. Given that aged animals were used in the present study, the modified RAM protocol described by Clelland et al. (2009) was used in water, drawing on aversive motivation, rather than appetitive motivation, to complete the task.

Similar to Clelland et al. (2009), the present study employed distinct cues on the walls around the maze, within the rat's visual range, in order to promote allocentric navigation. Allocentric navigation depends on distal cues, whereas egocentric navigation depends on proximal and internal cues. While egocentric and allocentric navigation have been shown to activate overlapping neural systems (Sherrill et al., 2013), the hippocampus seems to be preferentially involved in allocentric navigation, as evidenced by impairments in allocentric navigation following hippocampal lesions (McDonald & White, 1994). In addition, the hippocampus seems to be particularly involved during flexible utilization of a pre-existing allocentric representation (Zhang & Ekstrom, 2013). Therefore, distal cues were used to encourage hippocampus-dependent allocentric navigation. Random start locations for each trial were also used to prevent animals from using internal and proximal cues to follow a set route in order to reach the platform location (Devan, Goad, & Petri, 1996), further promoting hippocampus-dependent allocentric navigation.

Procedures

All behavioural testing began at approximately the same time for each day of

testing. The radial arm water maze consisted of a radial arm maze placed in a circular pool 153 cm in diameter. The radial arm maze was made of an inner circle 54.61 cm in diameter, with eight arms, 16.76 cm wide and 35.56 cm long, extending from the perimeter of the circle. The entrance of each arm was initially blocked off, with the exception of two arms. The pool was filled with water (23 ˚C ±1 ˚C) to 9 cm from the top of the arms and dark non-toxic tempera paint was used to make the water opaque. A platform measuring 14 cm x 12 cm was placed in one of the open arms, hidden approximately 1.5 cm below the surface of the water. Visual cues were presented on the walls of the room. Tracking software (*EthoVision XT* Video Tracking System) was used to measure and record the rats' movement in the water using a ceiling-mounted camera.

Rats were handled for three days prior to the start of the behavioural task, which lasted for 13 days. Rats were placed in the water, facing the end of a randomly pre-selected start arm that varied for each trial for a total of three trials per day on the first three days, followed by four trials per day for the following nine days, and three trials per day on the last day (Table 1). Each trial consisted of a sample phase, followed by a choice phase 20 seconds later. The interval between the sample and choice phases was increased to 60 seconds for the last six trials on the 12^{th} and 13^{th} days to increase cognitive demand. All rats underwent each trial before the next trial began in order to maximize the inter-trial interval.

Sample phase. During the sample phase, two of the eight arms were open – the start arm and the sample arm – while the entrances of the remaining arms were blocked. A platform was located in the sample arm. Upon being placed in the water in the start arm, facing the wall, the rat was given 60 seconds to locate the hidden platform in the sample arm. If the rat was unable to locate the platform in 60 seconds, the experimenter manually guided it to the platform. Upon

mounting the platform, the rat remained there for 10 seconds. The rat was then removed from the platform and placed in a covered holding cage for the specified delay interval (20 or 60 seconds).

Choice phase. Following the sample phase, the rat was placed back in the start arm for the choice phase. The choice phase differed from the sample phase in that three arms were open – the original start arm, the original sample arm, and a new choice arm, to which the platform had been moved. Rats were again given 60 seconds to locate the new location of the platform and were manually guided to it if they were unable to find it within the given time frame. In order to increase interference, the distance between the sample and choice arms varied from trial to trial (Clelland et al., 2009). The sample and choice arms were separated by a distance of two, three, or four arms with arms more closely presented in space thought to pose greater memory interference (Clelland et al., 2009).

Start, sample, and choice arms were randomly chosen from a pre-defined selection of configurations. Given that the total number of possible configurations for a spatial separation of two arms is greater than the total number of possible configurations for a separation of four arms, the start, sample, and choice arms were randomly chosen from a pre-defined selection of configurations. The pre-defined selection consisted of four possible configurations for each degree of spatial separation, for a total of 12 possible spatial configurations. Possible configurations were selected in a way that minimized the amount of times an individual arm was presented to the animal in a given day, such that no arm was over-presented relative to the other arms over the course of the study. Configurations were balanced in terms of whether the platform was located to the left or to the right of the platform, with either the sample or the choice arm being perpendicular to the start arm (Clelland et al., 2009). All rats underwent the first trial, consisting of the sample phase and the choice phase, before the second trial began. At the end of

the study, each rat was exposed to each of the three spatial separations a total of 16 times. On the 14th day, rats were placed back in the pool for a single sample phase to induce IEG expression. Three rats from each condition did not undergo the sample phase on this day and were anesthetized immediately upon removal from their home cages (cage controls). IEG data will be analyzed and discussed in future studies.

Histology

Forty-five minutes following the single sample phase, or upon removal from the home cage, rats were deeply anesthetized with isoflurane. Once completely unresponsive, rats were sacrificed by decapitation and brains were rapidly harvested and flash frozen in isopentane chilled in a mixture of 90% ethanol and dry ice. Eight brains were mounted with optimal cutting temperature (OCT) compound (Fisher Scientific, Whitby, ON) such that at least one treatment group and one cage control was present on each slide. Using a cryostat (Leica) at -20°C, coronal sections (20 μm) were obtained through the entire dorsal hippocampus and thaw-mounted on Superfrost Plus slides (VWR). Slides were dried and stored at -80°C.

Immunofluorescence Staining

BrdU and NeuN double-labeling. For each rat, 10 sections from the dorsal hippocampus [bregma -3.30 to -4.52 mm (Paxinos & Watson, 1986)] were randomly chosen. Sections were double-labelled for NeuN and BrdU as previously described (Lui et al., 2017). Slides were fixed in a 2% paraformaldehyde solution for 8 minutes and then washed three times in a 0.1M tris-buffered saline (TBS) solution. Sections were quenched in a 20% methanol and 0.6% H_2O_2 TBS solution for 20 minutes, followed by three washes with TBS. Sections were then blocked with tyramide signal amplification (TSA) blocking reagent (PerkinElmer, Boston, MA) for 30 minutes and incubated at 4°C with biotinylated mouse anti-NeuN antibody (1:2000; Millipore,

Temecula, CA) overnight. The next day, slides were rinsed once in TBS with 0.05% Tween 20 (TBS-T) and twice in TBS and then incubated for 30 minutes with VECTASTAIN ABC Kit (Vector Labs, Burlington, ON) and washed three times in TBS. Coumarin tyramide (PerkinElmer) was then applied for 40 minutes to visualize NeuN-labeled cells. Following one TBS-T and two TBS washes, sections were quenched with 1% H_2O_2 in saline sodium citrate (SSC) buffer for 20 minutes and washed in TBS another three times. Tissue sections were then blocked with mouse-on-mouse (M.O.M.) blocking reagent (Vector Labs) for one hour and washed in TBS and SSC buffer. Slides were then incubated in 50% formamide in SSC buffer at 65°C for two hours, washed in SSC buffer, and incubated in 2N HCl for 30 minutes at 37°C. Slides were then washed in 0.1M boric acid (pH 8.5) for five minutes to neutralize the sections. After six TBS washes, sections were incubated with monoclonal mouse anti-BrdU (1:100, Roche Applied Sciences, Montreal, QU) overnight at 4°C. The following day, after three TBS washes, sections were blocked with avidin/biotin blocking kit (Vector Labs) and incubated with biotinylated anti-mouse antibody (1:200, Vector Labs) for two hours. Slides were washed in TBS-T and TBS prior to being treated with VECTASTAIN ABC Kit for 40 minutes. In order to visualize BrdU-labeled cells, sections were treated with fluorescein (TSA kit labeling, PerkinElmer) for ten minutes following three TBS washes. Finally, sections were washed in TBS another three times, coverslipped with Vectashield mounting medium, and sealed with nail polish the following day.

Image Acquisition and Cellular Data Collection

Microscopy and cell counts were conducted blind. Manual z-stacked images were obtained using the 40x objective lens of a fluorescent microscope (Nikon, Canada). For each slide, image settings (e.g., exposure time, light intensity) were held constant. Images of the entire

DG were constructed with the stitching feature of the microscope using the 10x objective lens. Using NIS-Elements Imaging Software, the areas of the suprapyramidal and infrapyramidal blades of the DG were individually measured for each brain section and the number of double-labeled (BrdU and NeuN) cells were counted on each blade (Figure 2). Areas were multiplied by the tissue thickness (20 μm) to estimate the volume of each blade. The density of newborn neurons in each blade was calculated by dividing the total number of neurons on each blade by the estimated volume (Cai et al., 2012).

Statistical Analysis

All analyses were conducted using IBM SPSS statistical software. To analyze behavioural discrimination over the course of the RAWM, the average total number of errors made over 16 trials of each spatial separation was calculated. A 4x3 repeated measures ANOVA was conducted to compare errors made in each treatment group across all trials of the three spatial separations. Three separate one way ANOVAs for each spatial separation (S2, S3, S4) were also conducted, as well as a planned comparison using simple contrast to compare each aged group (old-enriched, old-exercise, old-controls) to the young controls. Pearson correlations were also calculated to assess the relationship between average number of wheel turns per day and number of errors made on the RAWM task at each spatial separation for the exercise group. The density of BrdU-labeled neurons in the suprapyramidal and infrapyramidal blades of the DG was analyzed using a 2x4 repeated measures factorial ANOVA. The level of statistical significance is set at $p \leq 0.05$.

Results

Radial Arm Water Maze (RAWM) Task

A 4x3 repeated measures factorial ANOVA to assess acquisition during the RAWM task

with a 20 second delay revealed a significant effect of spatial separation, $F(2, 56)=4.487$, $p=0.016$, but no treatment or interaction effect, $p>0.05$, suggesting that the number of errors made significantly varied by spatial separation for all rats regardless of treatment or age group (Table 2, Figure 3). Partial eta squared was used as a measure of effect size and a magnitude of 0.138 was found for spatial separation, suggesting that 13.8% of the variance found in number of errors made is attributable to separation. Post hoc tests using the Bonferroni correction revealed a significant difference in the number of errors made between S2 and S3, $p=0.025$ for all rats regardless of treatment or age group, with rats making significantly more errors at S2 compared to S3. No significant difference in the number of errors made was found between S3 and S4 or between S2 and S4. Cohen's d was also used as a measure of effect size for each comparison made (Table 3).

The one-way ANOVAs for errors made at each spatial separation revealed no significant main effect of group. Planned comparisons were conducted to compare each aged group with the young controls and revealed a significant difference between the number of errors made by the old control group compared to the young control group, only at the smallest spatial separation (S2), $t(28)=2.22$, $p=0.035$, Cohen's $d=-1.14$; no difference was found at the S3 or S4 separations (Figure 3). All of the effect sizes are noted in Table 4. No significant difference in number of errors made was found between the old-enriched and young group or the old-wheel and young group at any of the spatial separations (Figure 3). With a 60 second delay between the sample and choice phases, no significant difference was found between any of the four groups at any spatial separation (Figure 4).

For the exercise group, the relationship between task performance (number of errors made at each spatial separation on the RAWM) and the average number of wheel turns per day

over the course of the intervention was investigated (Table 5). While correlations between wheel turns and performance were weak at S2 ($r=-0.140$, $p=0.765$) and S3 ($r=-0.139$, $p=0.767$), a moderate negative correlation was found at S4 ($r=-0.450$, $p=0.311$). Results suggest that at the easiest level of the task, a moderate inverse relationship exists between task performance and wheel running, such that rats with a higher average of wheel turns per day made fewer errors on the RAWM at this spatial separation.

The behavioural results therefore suggest that when encountering a high interference memory task, aged rats struggle to accurately discriminate between similar locations in comparison to young controls, but that long-term exercise or environmental enrichment seem to be protective against such deficits.

Quantification of Adult Hippocampal Neurogenesis

A 2x4 repeated measures ANOVA was conducted to compare the density of new neurons in the suprapyramidal and infrapyramidal blades of the DG among the four treatment groups (Table 6). This analysis revealed a significant blade effect, $F(1, 28)=19.667$, $p<0.001$, $\eta^2=0.413$, such that the infrapyramidal blade had a higher density of BrdU-labeled neurons, as has been previously reported (Satvat, Gheidi, Voll, Odintsova, & Marrone, 2012). A significant blade by treatment interaction effect was also found, $F(3, 28)=63.481$, $p<0.001$, $\eta^2=0.872$, such that the effect of blade depended on age (i.e., a difference in the density of BrdU-labeled neurons between blades was evident in the young group, but not in the aged groups) (Figure 5; Table 7).

To compare the densities of BrdU-labeled neurons in each blade of the DG separately, two one-way ANOVAs were conducted followed by post hoc comparisons. The one-way ANOVA for cells double-labelled with BrdU and NeuN in the suprapyramidal blade of the DG revealed a significant main effect of group, $F(3, 31)=36.242$, $p<0.001$, $\eta^2=0.795$ (Figure 5; Table

8). Results indicate that the number of new neurons significantly differed between one or more of the treatment groups, with treatment explaining 79.5% of the variance found in number of new neurons in the suprapyramidal blade. A post hoc comparison using the Games-Howell correction (Field, 2013) revealed a significant difference in the density of new neurons between the young control group and all other treatment groups, $p<0.001$, but no significant difference was found between any of the aged groups.

A one-way ANOVA was similarly conducted for new neurons in the infrapyramidal blade of the DG. Due to a violation of the assumption of the homogeneity of variances ($p<0.05$), a corrected Welch F value was calculated (Field, 2013). Results indicated a significant difference between treatment groups, Welch's $F(3, 14.407)=38.106$, $p<0.001$ (Figure 5; Table 8), with treatment group explaining 89.8% of the variance in number of new neurons in the infrapyramidal blade, $\Pi^2=0.898$. Similar to the suprapyramidal blade, a Games-Howell post hoc test revealed a significant difference in the density of new neurons in the infrapyramidal blade between the young control group and all other treatment groups, $p<0.001$, but no significant difference was found between any of the aged groups. Cellular results therefore suggest that aging has a major impact on the long-term survival of adult-born neurons, regardless of behavioural intervention

Discussion

The process of normal aging is associated with suppression of adult hippocampal neurogenesis (McDonald & Wojtowicz, 2005), which has been suggested to be related to poor performance on high interference memory tasks (Clelland et al., 2009). Voluntary exercise and environmental enrichment have been shown to stimulate hippocampal neurogenesis (Creer et al., 2010; Kempermann et al., 2002) and potentially alleviate some of the memory deficits

experienced with normal aging (Kempermann et al., 2002). Therefore, in the present study, an animal model was used to assess: 1) whether adult hippocampal neurogenesis and the ability to overcome interference on a behavioural memory task are negatively affected by the normal aging process, 2) whether long-term exposure to voluntary exercise or environmental enrichment can enhance performance on a high interference memory task, and 3) whether these interventions have an effect on the long-term survival of adult born neurons. To address these objectives, young rats housed in standard conditions and old rats randomized into one of three housing conditions (standard, exercise, or enriched environment) were tested on a DNMP RAWM task with a high interference component. Rats were injected with BrdU to label newborn neurons on the first six days of the intervention, which lasted for ten months, and were sacrificed following 13 days of the RAWM task (a total of 16 trials per spatial separation).

Memory Interference Reduction

The ability to overcome memory interference has been thought to be associated with adult hippocampal neurogenesis (Clelland et al., 2009; Sahay et al., 2011), which has consistently been shown to be suppressed with age (McDonald & Wojtowicz, 2005). In addition, exercise and environmental enrichment have been suggested to increase adult hippocampal neurogenesis (Creer et al., 2010; Kempermann et al., 1998). Given the evidence implicating adult-generated neurons in memory interference reduction (Clelland et al., 2009), one would expect that animals with the most adult-generated neurons would perform better on the high memory interference DNMP RAWM task compared to animals with less adult-generated neurons. The results of the present study confirm that the process of normal aging does indeed result in a reduced ability to overcome memory interference, such that aged controls made more errors on the RAWM task specifically when memory interference was the highest (S2).

Furthermore, exercise and environmental enrichment seem to be capable of having a protective effect in aged animals at the same level of memory interference. However, whether performance on the RAWM task in each of the four groups correlates with neurogenesis levels is not something that could be assessed in the present study. Mature adult-born neurons function in much the same way as developmentally generated GCs, no longer exhibiting the characteristics of immature neurons that make them well-suited to reduce interference (Esposito et al., 2005; Ge, Yang, Hsu, Ming, & Song, 2007; Laplagne et al., 2007; Schmidt-Hieber et al., 2004). Therefore, the cohort of neurons labeled with BrdU in the present study counted at ten months post-injection likely did not play a role in reducing interference on the RAWM task. However, the present study cannot rule out the possibility that new neurons generated closer to the day of sacrifice may have helped improve performance on the RAWM in the intervention groups when interference was highest.

When number of errors made was assessed over the course of all trials of all spatial separations, spatial separation significantly influenced the number of errors made, but no difference was found between treatment groups, suggesting that all animals were able to similarly learn the RAWM task following 16 trials of each spatial separation. However, difficulty overcoming interference was observed in aged rats compared to young controls as indicated by a greater number of errors made specifically when the spatial separation between the sample and choice arms was smallest. Such a deficit was not evident in the aged rats given either exercise or environmental enrichment compared to young controls, suggesting a likely protective effect of these interventions when interference is highest. In the exercise group, weak correlations were found between average number of wheel turns per day and performance on the RAWM at spatial separations of S2 and S3. Interestingly, at S4, a moderate inverse relationship

was found (r=-0.450). While performance at S2 and S3 was found to be weakly related to how much an animal ran, exercise increased performance of aged animals at S2 to the level of young controls, suggesting that any exposure to exercise seems to be beneficial for task performance. However, at S4, amount of running was moderately related to performance such that the more an animal ran per day, the less errors it made on the RAWM specifically when interference was lowest. When the interval between the sample and choice phases was increased to 60 seconds, there was no significant difference in errors made between any of the treatment groups at any of the spatial separations, suggesting that this interval increased cognitive demand for all animals, making it equally difficult for all groups to perform.

A common characteristic of tasks in which adult-generated neurons have been shown to be involved is a high memory interference component. Thus, the ability to overcome memory interference has often been associated with higher levels of hippocampal neurogenesis (Clelland et al., 2009; Sahay et al., 2011). The process by which adult-born GCs are involved in memory interference reduction is still not clear, although a pervasive view in the literature is that these neurons are able to reduce interference by engaging in pattern separation. However, a recent review of the evidence suggests that the characteristics of adult-generated neurons do not make them suited to directly perform the function of pattern separation (Becker, 2017). Specifically, computational models have recently revealed that models that are more highly active correspond to a higher probability of overlap in neural codes for distinct memories. However, neural models characterized by sparse coding are less likely to have overlapping patterns of activation (i.e., more pattern separation) (Finnegan & Becker, 2015). Given that at the age of 4-6 weeks, adult-generated neurons do not fire sparsely, but rather are hyperactive (Schmidt-Hieber et al., 2004), a direct role for these new neurons in pattern separation is improbable. Furthermore, computational

models designed to assess how adult-born neurons specifically are involved in the learning of novel overlapping patterns revealed that models possessing both young and old neurons outperformed models lacking neurogenesis (Finnegan & Becker, 2015). Interestingly, models that lacked neurogenesis learned sparser, less overlapping codes than models with both young and old neurons. In fact, the addition of new neurons seemed to lead to a reduction in pattern separation. Computational models have therefore supported the notion that a combination of young neurons with high firing rates and mature neurons that fire more sparsely results in more accurate pattern encoding compared to models that rely solely on sparse coding, despite the fact that such models reduce pattern separation. Therefore, while new neurons do seem to contribute to memory interference reduction, they do not appear to do so by engaging directly in pattern separation (Finnegan & Becker, 2015).

While the hyperexcitability of immature neurons likely precludes them from a direct role in pattern separation, research has suggested that they may reduce memory interference by influencing pattern separation indirectly. Immature neurons experience a period of high synaptic plasticity and a low threshold of activation, as well as low input specificity before maturing around 6-8 weeks of age and becoming more similar to developmentally generated neurons (Esposito et al., 2005; Ge et al., 2007; Laplagne et al., 2007; Schmidt-Hieber et al., 2004). In an effort to balance overall DG activity levels (Meltzer, Yabaluri, & Deisseroth, 2005), highly active young neurons may exert an inhibitory effect on mature developmentally generated cells, leading to increased sparse coding and ultimately increased pattern separation (Becker, 2017). Specifically, as a result of their high excitability, young adult-born GCs are able to respond to weak inputs, such as subtle changes in context, and exert feedback inhibition, either directly through local inhibitory neurons or indirectly through mossy cells, on mature GCs (Sahay,

Wilson, & Hen, 2011). Altering the rate of neurogenesis, either by upregulation through genetic manipulation or by ablation through focal x-irradiation, has been shown to decrease or increase the strength of neuronal activation in the DG, respectively (Ikrar et al., 2013). Changes in excitability of the DG further suggest that adult hippocampal neurogenesis alters the sparseness of activity in the DG, which may indirectly influence pattern separation (Ikrar et al., 2013). In addition, neurogenesis increases the number of available encoding units, thereby increasing competition for synaptic inputs. Neurogenesis may thus enhance the capacity of the DG to encode new information and minimize interference by extracting distinct features of similar stimuli (McAvoy, Besnard, & Sahay, 2015). This notion further supports an indirect role for new neurons in pattern separation.

Another feature of adult-generated neurons that makes them well-suited to reduce memory interference is their sparse connectivity, meaning they receive few inputs from the EC and CA3 (Bergami et al., 2015). The sparse connectivity of adult-generated neurons has been suggested as an alternate mechanism by which they reduce memory interference, allowing them to respond to two similar stimuli by creating two overlapping but distinct patterns of activation (Becker, 2017). Specifically, sparse connectivity, in combination with high excitability and low inhibition relative to excitation, prevents immature neurons from responding broadly to a wide range of stimuli, such that immature neurons may respond strongly to select inputs while their more densely innervated mature counterparts are less responsive to a broader range of cortical inputs (Dieni, Nietz, Panichi, Wadiche, & Overstreet-Wadiche, 2013). The characteristics of immature neurons therefore make them capable of decreasing pattern separation while simultaneously reducing memory interference.

The cohort of adult-generated neurons labeled in the present study likely was not involved in the RAWM task, given that, at ten months of age, these neurons function much the same way as developmentally generated neurons. Thus, at the time of the RAWM task, the labeled cohort no longer exhibited the characteristics of immature neurons, such as sparse coding and sparse connectivity, which make them well-suited to reduce memory interference. However, aged animals exposed to exercise or environmental enrichment appeared to perform similarly to young controls at the highest level of interference (S2) in the RAWM. Therefore, although not labeled in the present study, it is likely that immature neurons generated closer to the day of sacrifice and capable of overcoming memory interference were involved in improving performance on the task – a possibility that should be further investigated in future studies.

Long-term Survival of BrdU-labeled Neurons

The survival of BrdU-labeled neurons was examined ten months following the start of the intervention. Results of the present study revealed a significant difference between the young control group and the three aged groups, with young controls possessing a greater density of BrdU-labeled neurons at ten months post-injection. No difference in the long-term survival of neurons was found between aged control animals and those housed in either intervention condition.

Existing research has shown that neurons generated in the adult rat DG undergo a period of apoptosis between 6 and 28 days post-injection. During this period, the GC layer undergoes a decrease of approximately 50% in the number of BrdU-labeled cells (Dayer et al., 2003). Following this initial period of cell death, surviving BrdU-labeled cells in the rat DG have been shown to persist in the GC layer for at least six months post-injection, with no significant change in number of cells from one month to six months post-injection (Dayer et al., 2003). Given the

stability of adult-born neurons between this time period, it is thought that these neurons are likely to persist past six months of age. In fact, in mice, no significant difference was found between the number of BrdU-labeled neurons at four weeks and 11 months post-injection (Kempermann et al., 2003), indicating the stability of adult-generated neurons that survive beyond four weeks. However, to the extent of our knowledge, the long-term survival of adult-generated neurons under the influence of environmental enrichment and exercise during the normal aging process has not been assessed. Results of the present study suggest that environmental conditions do not influence the long-term survival of adult-born neurons, in spite of prior research that appears to suggest otherwise. Prior research investigating the effect of long-term exposure to environmental enrichment on neurogenesis found a significant survival-promoting effect of the intervention in older mice (Kempermann et al., 2002). Ten-month old mice exposed to the enriched environment for ten months were found to have a significantly greater number of BrdU- and NeuN-positive cells, suggesting that long-term enrichment offers a significant survival-promoting effect on new neurons generated in older age (Kempermann et al., 2002). However, it is important to note that animals were injected with BrdU during the last twelve days of the ten months of the intervention and were sacrificed 28 days later. Thus, the survival of adult-born neurons under the influence of long-term environmental enrichment could not be assessed past four weeks of age, in spite of the ten-month duration of the intervention. The results of the present study therefore do not contradict existing research, but rather indicate that environmental enrichment may not provide a persistent survival-promoting effect on adult-generated neurons. This notion is further supported by findings that suggest that short- and long-term exposure to environmental enrichment do not have the same effect on neurogenesis. In a study where interventions lasted for six months, a significantly greater number of BrdU-positive cells at one day post-injection

was found in mice housed in an enriched environment for 68 days followed by standard housing compared to those housed in long-term enrichment or in control conditions for the duration of the study (Kempermann & Gage, 1999). Following an additional four weeks, no significant difference in the number of BrdU and NeuN double-labeled cells between the three groups was found (Kempermann & Gage, 1999). Due to a lack of a difference between the long-term enrichment and the control groups, the authors suggest that continued environmental enrichment may result in a reduced capacity of the stimuli to elicit a neurogenic response (Kempermann & Gage, 1999). The authors further suggest that stimulation of neurogenesis may not be a result of exposure to complex stimuli, but rather to the novelty of the stimuli (Kempermann & Gage, 1999), which could potentially explain why the present study did not find a lasting effect of environmental enrichment on long-term survival of adult-born neurons. While stimuli in the enriched group was changed regularly every few days, some of the toys that were provided were similar in shape or material and were presented to the animals multiple times over the duration of the intervention. Thus, while exposure to these complex stimuli continued, their novelty may have worn off, potentially reducing their survival-promoting effect.

Another possible explanation for the absence of a survival-promoting effect of enrichment in the present study is that the stimulatory effect of environmental enrichment may be dependent on the proliferative capacity of precursor cells. Animals that were exposed to exercise followed by a period of environmental enrichment possessed a significantly greater number of BrdU-labeled neurons in comparison to animals exposed to exercise followed by standard conditions or in standard conditions followed by enrichment (Fabel et al., 2009). In addition, no significant difference was found between the standard/enriched group and the control group, suggesting the effect of environmental enrichment may depend on a prior priming

of proliferative precursor cells (Fabel et al., 2009). As such, in the present study, environmental enrichment may not have been sufficient to elicit any long-term changes in adult neurogenesis given the absence of an initial pro-proliferative stimulus such as exercise.

The physiological changes that occur following exposure to exercise or environmental enrichment may explain the difference in the proliferation-promoting capacity of each of these interventions. While both voluntary exercise and environmental enrichment have been shown to increase spine density and to induce expression of similar trophic factors (for review, see Olson, Eadie, Ernst, & Christie, 2006), exercise has been shown to have distinct effects that may contribute to the proliferation of neural progenitor cells. For example, voluntary exercise induces changes to the vasculature of the brain, whereas the research on environmental enrichment thus far has not found such an effect. Physical activity has been shown to be associated with increased cerebral blood flow (Yancey & Overton, 1993) and volume (Swain et al., 2003), as well as increased blood-brain-barrier (BBB) permeability (Sharma, Cervós-Navarro, & Dey, 1991). Furthermore, physical activity increases angiogenesis (Black, Isaacs, Anderson, Alcantara, & Greenough, 1990; Isaacs, Anderson, Alcantara, Black, & Greenough, 1992; Kleim, Cooper, & VandenBerg, 2002; Swain et al., 2003), as well the utilization of glucose (Vissing, Andersen, & Diemer, 1996). Given the increase in circulation in response to physical activity, the hippocampus may have greater access to certain neurotrophic factors that promote cell proliferation as a result. For example, vascular endothelial growth factor (VEGF) has been shown to stimulate neuronal precursor cells both in vitro and in vivo (Jin et al., 2002). Specifically, VEGF stimulated cell proliferation, but had no effect on cell survival. VEGF also has an angiogenic effect, stimulating the formation of new blood vessels (Leung, Cachianes, Kuang, Goeddel, & Ferrara, 1989; Senger et al., 1983). Given that VEGF has been shown to

increase in humans as a result of exercise, it may play a role in the effect of physical activity on cell proliferation. In a study where a VEGF antagonist that is impermeable to the BBB was used, exercise was not shown to have an effect on neural precursor proliferation, further suggesting that VEGF may play a critical role in the induction of adult hippocampal neurogenesis as a result of exercise (Fabel et al., 2003).

Insulin-like growth factor 1 (IGF-1) and fibroblast growth factor 2 (FGF-2) have also been suggested to be potential mechanisms by which exercise induces cell proliferation in the DG. Similar to VEGF, preventing IGF-1 circulation in the brain resulted in the inhibition of exercise-induced increases in neurogenesis (Trejo, Carro, & Torres-Aleman, 2001). While such an effect has not yet been demonstrated for FGF-2, exercise has been shown to result in increased expression of FGF-2 in the hippocampus (Gómez-Pinilla, Dao, & So, 1997). Furthermore, co-application of FGF-2 and IGF-1 has been shown to produce maximal proliferation in the DG (Aberg et al., 2003), suggesting a potential role for both of these factors in exercise-induced neurogenesis.

While prior research has clearly demonstrated the effect of exercise on neurogenesis, the present study found that, similar to environmental enrichment, aged animals with access to a running wheel did not show an increase in the number of BrdU-labeled neurons compared to controls. Prior studies have shown that precursor cells in the DG remain capable of activity-induced proliferation even in older age (Kronenberg et al., 2006). However, this stimulatory effect seems most prominent following acute exposure to physical activity. Specifically, mice exposed to either 3, 10, or 32 days of voluntary exercise showed a significant difference in the number of BrdU-positive cells at one day post-injection, such that continued exposure resulted in less labeled cells, suggesting a reduction in the pro-proliferative effect of exercise (Kronenberg

et al., 2006). Interestingly, while less BrdU-positive cells were present following 32 days of exercise compared to 3 days, the researchers found an increase in the number of cells expressing DCX and calretinin (CR), both markers of immature neurons, as a result of the acute stimulation of proliferating precursor cells (Kronenberg et al., 2006). However, it is important to note that not all immature cells expressing DCX fully develop into mature neurons (Kempermann et al., 2003). While animals were exposed to exercise for six months, they were not injected with BrdU until one day before perfusion. Therefore, the long-term survival of DCX- and CR-positive cells could not have been assessed. Following chronic exposure to voluntary exercise, an age-dependent decline in cell proliferation was not evident. In spite of such an effect, no difference was observed in the number of post-mitotic (DCX- or CR-positive) cells or in the total number of GCs in the DG, indicating no difference in net neurogenesis following long-term physical activity compared to controls (Kronenberg et al., 2006). Similar to the findings of Fabel et al. (2009), the effect of exercise on neurogenesis seems to be pro-proliferative, increasing the potential for new neurons to be added to the functional network of the hippocampus. However, given the absence of cognitive stimulation, the potential of these immature cells may not be realized and may not result in a sustained effect on net neurogenesis (Kronenberg et al., 2006). Given that research thus far has shown that exercise primarily promotes cell proliferation and the short-term survival of new neurons (van Praag et al., 1999), it may be that voluntary running in the present study did in fact increase cell proliferation, but, in the absence of an additional survival-promoting stimulus such as enrichment (Nilsson et al., 1999), did not result in any long-term changes in the survival of these immature cells, resulting in no difference in the density of BrdU-labeled neurons compared to old controls at 10 months post-injection.

The results of the present study therefore indicate that, as hypothesized, aging does result in a significant reduction in the density of adult-generated neurons at 10 months post-injection, as evidenced by the significant difference between young and old rats. Furthermore, given the absence of a difference between the aged control group and the two intervention groups, the results indicate that long-term exercise and environmental enrichment do not elicit changes in the density of adult-generated neurons in aged animals at 10 months post-injection. Long-term behavioural interventions therefore do not seem to offer a long-term protective effect against age-related changes in hippocampal neurogenesis.

One of the primary objectives of the present study was to examine the long-term survival of adult-generated neurons and the influence of long-term behavioural interventions on this phase of neurogenesis. As such, BrdU injections began on the first day of the animals' respective housing conditions, labeling replicating cells that had been in the S phase of the cell cycle on those days. Immunofluorescence staining for BrdU in combination with NeuN allowed us to identify differentiated neurons that had been generated in adulthood on the day of injection, ten months prior to sacrifice. Importantly, GCs generated in adulthood follow a developmental trajectory and a pattern of neuronal integration similar to that of neurons generated prenatally (Esposito et al., 2005). Upon reaching maturation, adult-generated GCs exhibit high input specificity, lower excitability, and lower synaptic plasticity (Ge et al., 2007). Prior research has also identified a functional convergence of adult-born and developmentally generated GCs, as measured by a variety of parameters involving glutamatergic and GABAergic (gamma-aminobutyric acid) afferents, as well as intrinsic membrane properties. Such a convergence suggests that adult-born neurons do not mature to form a functionally distinct population, but rather are recruited into the existing functional population (Laplagne et al., 2007). Given that

mature adult-born neurons function in much the same way as developmentally generated GCs, they no longer exhibit the characteristics of immature neurons that make them well-suited to reduce interference. Thus, the ten-month-old BrdU-labeled neurons counted in the present study likely did not play a role in overcoming memory interference on the RAWM task.

Although outside of the scope of the present study, labeling cells with other markers such as DCX or CR would allow one to further elucidate the role of adult-generated neurons in interference reduction. DCX is expressed by newly replicated GCs when they are at a stage between lineage-determined precursor cells and immature neurons in the early post-mitotic stage (Rao & Shetty, 2004). Upon further maturation, cells begin to express CR, indicating they are in the early post-mitotic stage of GC development (Brandt et al., 2003), before fully maturing and expressing NeuN around four to six weeks of age (Brown et al., 2003; Kempermann et al., 2003; McDonald & Wojtowicz, 2005; Snyder et al., 2009). Given that adult-generated cells in the rat DG express peak levels of DCX when they are one to two weeks of age (Snyder et al., 2009), immunofluorescence staining to label immature neurons expressing DCX in the present study would allow one to identify cells that were generated just before the RAWM task (approximately two weeks pre-sacrifice) and are thus likely to be engaged in interference reduction. However, prior research suggests that there may not be a difference in the density of DCX-positive neurons between groups following the long-term interventions that were provided. Specifically, six months of voluntary exercise has not been found to significantly increase the number of DCX- or CR-positive cells in the DG (Kronenberg et al., 2006). Similarly, six months of environmental enrichment did not significantly increase the number of BrdU-positive cells at one day post-injection or the number of BrdU/NeuN double-labeled cells four weeks post-injection (Kempermann & Gage, 1999). Regardless, quantifying a younger cohort of neurons to assess

their role in interference reduction, particularly following long-term behavioural interventions, may be of interest for future research.

Concluding Remarks

Results of the present study indicate that the ability to overcome high interference on a behavioural memory task is negatively affected by the process of normal aging. Older animals experienced greater difficulty overcoming interference on the DNMP RAWM task compared to young controls. In addition, results also confirm that neurons generated in adulthood in the DG are capable of surviving for at least ten months; however, aging results in a significant reduction in the density of BrdU-labeled neurons that survive to this point. Adult hippocampal neurogenesis therefore seems to be significantly suppressed in older animals compared to younger animals. Interestingly, long-term exposure to environmental enrichment or exercise seems to influence performance on the high interference memory task, but did not influence the long-term survival of adult-generated neurons. Environmental enrichment may not have resulted in changes in the long-term survival of adult-born neurons as it is possible that the novelty of the enrichment stimuli wore off over the duration of the intervention (Kempermann & Gage, 1999). Prior research has also suggested that the survival-promoting effect of environmental enrichment may depend on the proliferative capacity of precursor cells (Fabel et al., 2009), which may not have been sufficiently promoted by enrichment alone. Additionally, exercise may have resulted in an increase in the proliferation of precursor cells (Kronenberg et al., 2006), but, in the absence of an additional survival-promoting stimulus, did not result in any changes in the long-term survival of adult-born neurons. While the involvement of BrdU-labeled neurons in the RAWM task was not directly investigated in the present study, it is possible that new neurons generated throughout the duration of the study, closer to the day of sacrifice, may have aided performance.

Regardless, the results of the present study suggest that long-term exposure to enrichment or exercise may influence performance on a high interference memory task. Although the mechanism by which these interventions reduce interference remains to be further investigated, it is possible that highly excitable immature neurons engage in interference reduction by exerting an inhibitory effect on mature GCs or as a result of their sparse connectivity (Becker, 2017). While outside the scope of the present study, future research should aim to further address this issue. Regardless, the results of the current study indicate that when interference is highest, aged animals experience a deficit in reducing interference compared to young animals, but long-term exposure to exercise and environmental enrichment may offer a protective effect. The present findings may have implications for elderly populations, given that behavioural interventions implemented in middle age seem to be capable of preserving cognitive abilities later in life.

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Table 1

Number of Trials and Length of Interval on Each Day of RAWM Testing

Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**12**	**13**	**14**
Number of Trials	3	3	3	4	4	4	4	4	4	4	4	1	3	3	1
Interval (seconds)	20	20	20	20	20	20	20	20	20	20	20	20	60	60	Sample phase only

Note. Interval indicates number of seconds between sample and choice phases during each trial of the RAWM.

Table 2

Mean Number of Errors Made in the RAWM Task with a 20 s Delay

	Number of Errors		
Treatment Group	**S2**	**S3**	**S4**
Old	19.86 ± 1.45	14.43 ± 1.01	14.57 ± 1.12
Old-Enriched	18.00 ± 1.30	13.33 ± 0.83	15.89 ± 0.95
Old-Wheel	17.00 ± 1.30	13.14 ± 0.92	16.57 ± 1.07
Young	13.78 ± 0.91	13.78 ± 0.77	16.11 ± 0.90

Note. Data are mean ± SEM. n=7 for Old and Old-Wheel and n=9 for Young and Old-Enriched.

Total of 16 trials per rat per spatial separation.

Table 3

Effect Sizes for Number of Errors Made in the RAWM Between Each Spatial Separation

Contrast		**p-value**	**Cohen's d**
S2	S3	0.025	-0.68
S2	S4	0.900	-0.24
S3	S4	0.109	0.59

Table 4

Effect Sizes for Number of Errors made in the RAWM for Each Aged Group Compared to Young Controls

Spatial Separation	Contrast	p-value	Cohen's d
S2	Old	0.042	-1.14
	Old-Enriched	0.125	-1.10
	Old-Wheel	0.268	-0.62
S3	Old	0.743	-0.15
	Old-Enriched	0.810	0.12
	Old-Wheel	0.749	0.15
S4	Old	0.445	0.40
	Old-Enriched	0.906	0.05
	Old-Wheel	0.818	-0.13

Table 5

Effect Size Correlations for Average Wheel Turns and Number of Errors Made on the RAWM

Spatial Separation	**Effect Size Correlation (r)**	**p-value**
S2	-0.14	0.765
S3	-0.14	0.767
S4	-0.45	0.311

Note. Wheel turn counts represent the average number of wheel turns made per animal per day over the course of the intervention. Wheel turns were correlated with the total number of errors made by each rat on the RAWM at each spatial separation.

Table 6

Mean Cell Densities of the Suprapyramidal and Infrapyramidal Blades of the DG

Cellular Densities					
Area	**Young**	**Old**	**Old-Wheel**	**Old-Enriched**	**p-value**
Suprapyramidal Density	$3.03 \times 10^6 \pm 2.60 \times 10^7$	$9.36 \times 10^7 \pm 1.58 \times 10^7$	$7.94 \times 10^7 \pm 1.65 \times 10^7$	$8.71 \times 10^7 \pm 1.04 \times 10^7$	<0.001
Infrapyramidal Density	$4.76 \times 10^6 \pm 3.26 \times 10^7$	$9.92 \times 10^7 \pm 1.26 \times 10^7$	$9.65 \times 10^7 \pm 1.72 \times 10^7$	$1.10 \times 10^6 \pm 9.49 \times 10^8$	<0.001

Note. Data are mean ± SEM. n=7 for Old and Old-Wheel and n=9 for Young and Old-Enriched.

Table 7

Effect Sizes for Density of BrdU-labeled Neurons Between Suprapyramidal and Infrapyramidal Blades of the DG per Treatment Group

Treatment Group	**Cohen's d**
Old	0.15
Old-Enriched	0.75
Old-Wheel	0.38
Young	1.95

Table 8

Effect Size for Density of BrdU-labeled Neurons Between Treatment Groups per Blade of the DG

	Contrast		**Cohen's d**
Suprapyramidal Blade	Old	Young	3.23
	Old-Enriched	Young	3.64
	Old-Wheel	Young	3.42
	Old	Old-Enriched	-0.18
	Old	Old-Wheel	-0.33
	Old-Wheel	Old-Enriched	0.21
Infrapyramidal Blade	Old	Young	4.89
	Old-Enriched	Young	5.08
	Wheel	Young	4.76
	Old	Old-Enriched	0.34
	Old	Old-Wheel	-0.07
	Old-Wheel	Old-Enriched	0.36

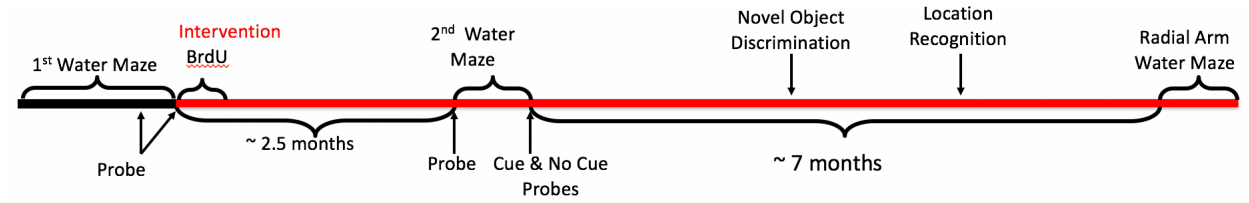

Figure 1. Timeline of procedures.

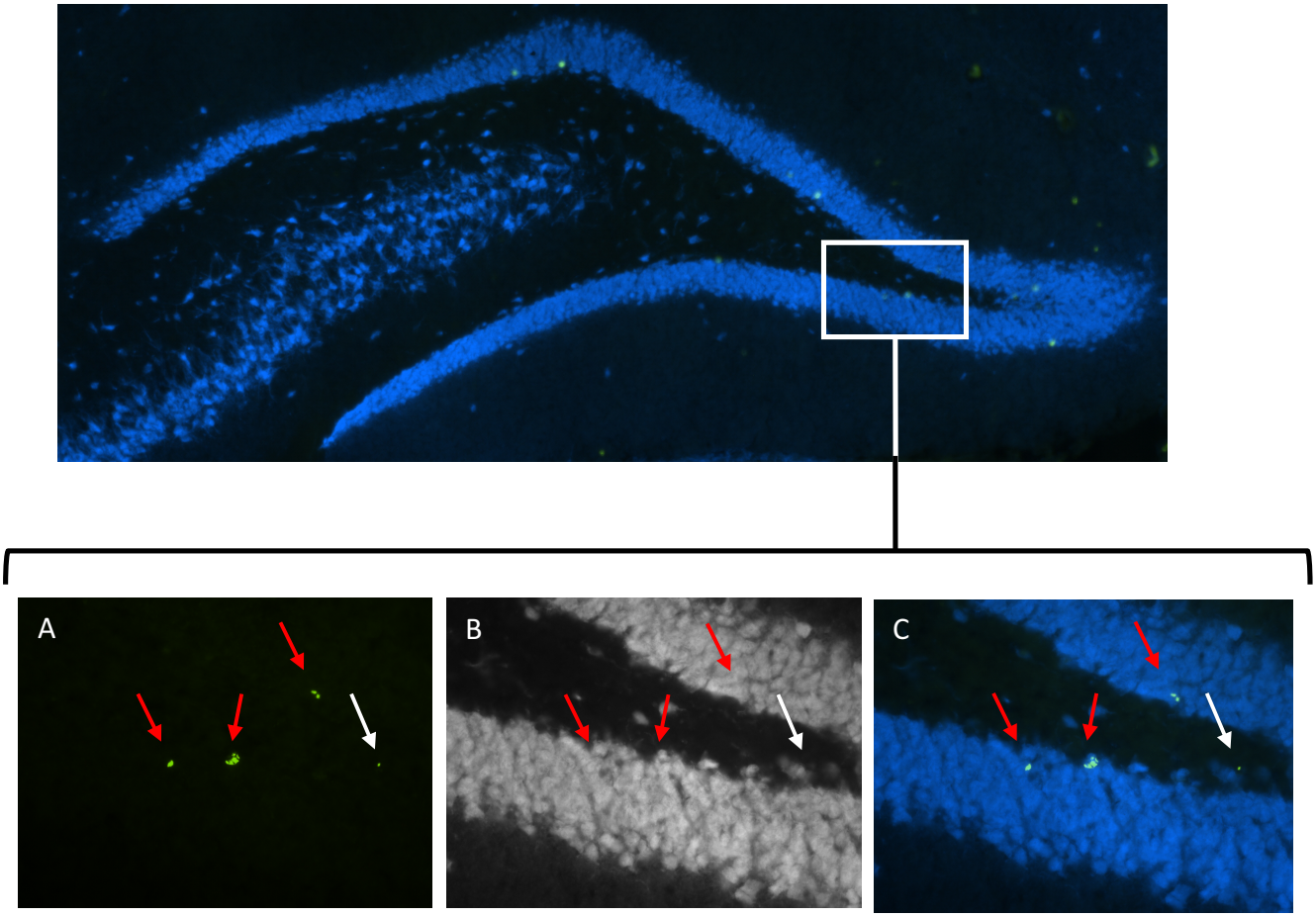

Figure 2. Representative fluorescent microscope image of the DG (top) with BrdU-positive cells (green) taken with a 10x objective. Images below represent the quantification of new neurons using a 40x objective. BrdU-labeled cells (A) overlap with NeuN-labeled cells (B; red arrows) thus labeling adult-generated neurons (C; red arrows). A BrdU-labeled cell that is not double labeled with NeuN, and thus is not a neuron, is indicated by a white arrow.

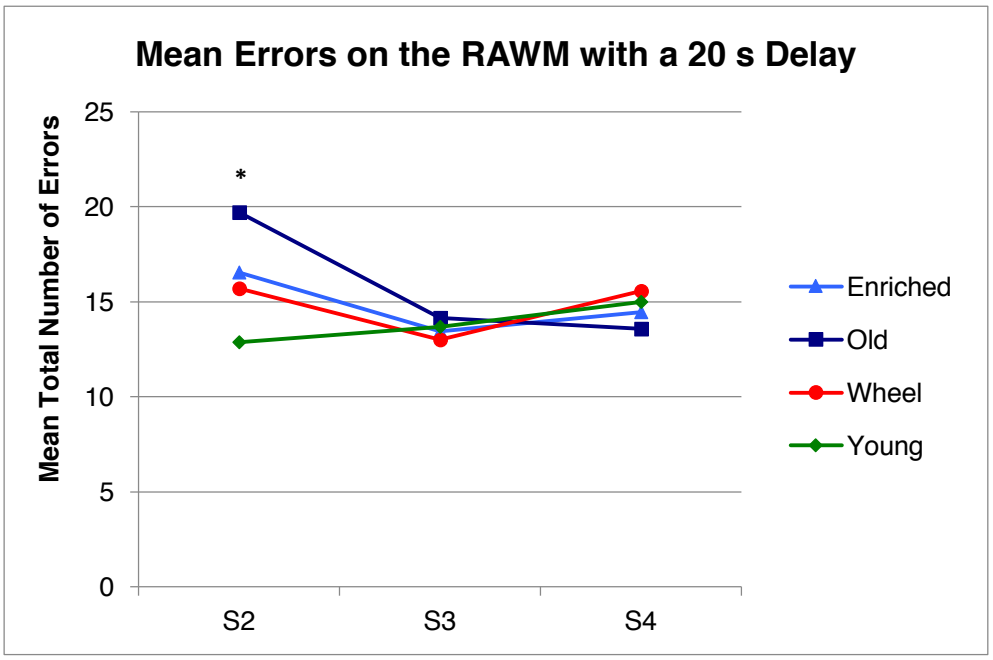

Figure 3. Mean total number of errors made by Old, Enriched, Wheel, and Young groups over the course of the experiment, with a 20 second delay between the sample and choice phases. Data are mean ± SEM. *p≤0.05. A significant difference was found only between the Young and Aged groups at S2, t(28)=2.22, p=0.035.

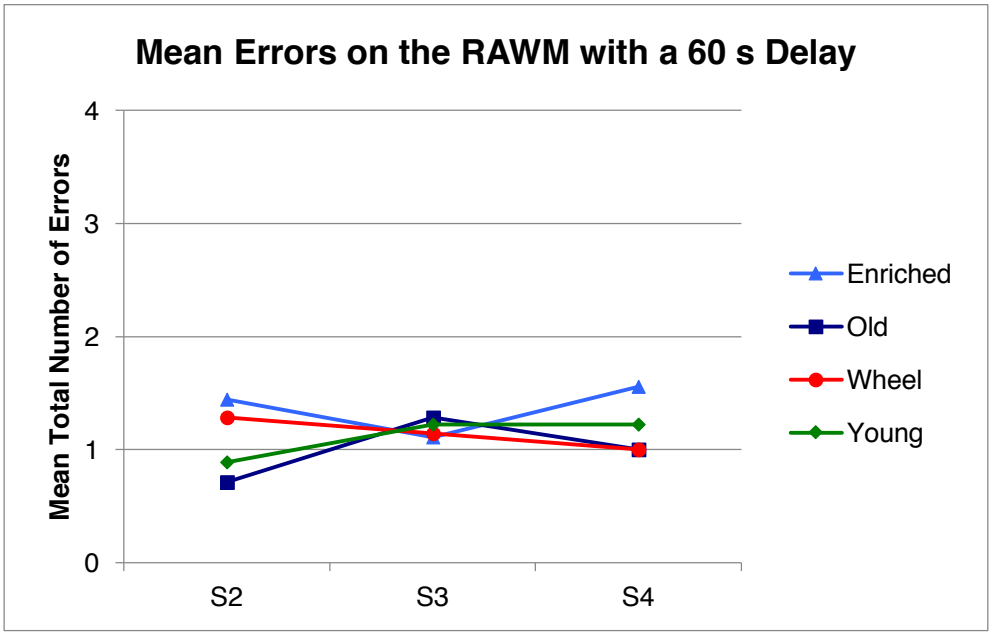

Figure 4. Mean total number of errors made by Old, Enriched, Wheel, and Young groups over the course of the experiment, with a 60 second delay between the sample and choice phases. Data are mean ± SEM. No significant differences were found between groups at any spatial separation ($p>0.05$).

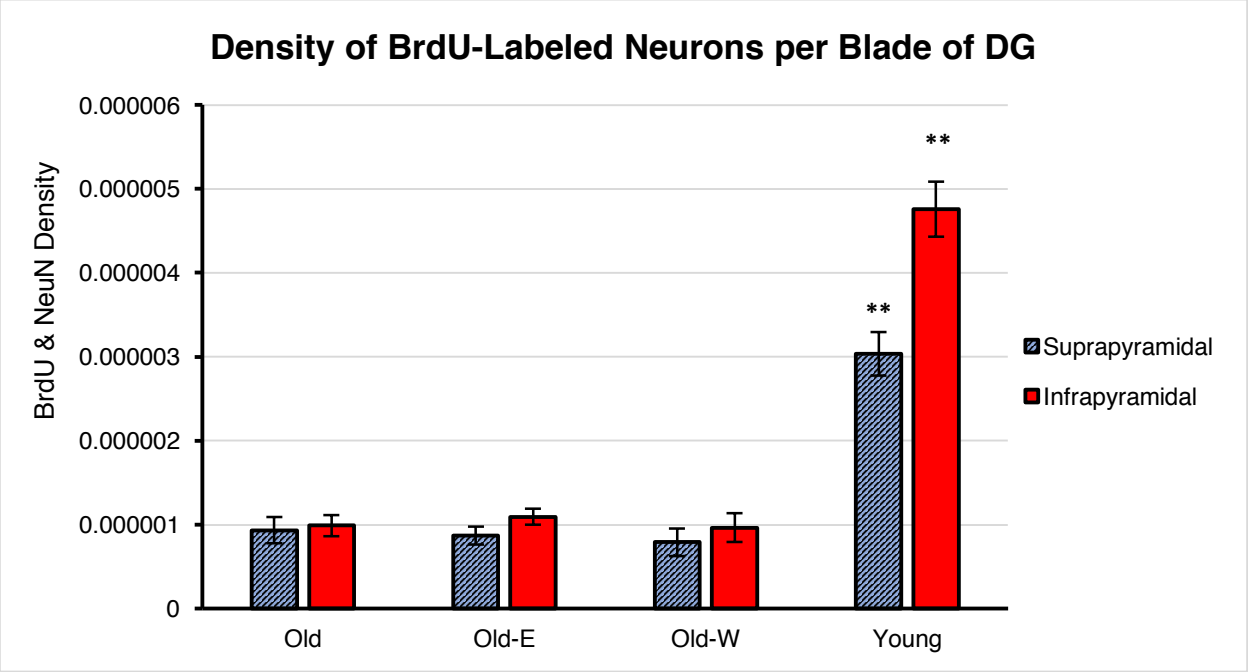

Figure 5. The density of newborn neurons in the suprapyramidal and infrapyramidal blades of the DG in each treatment group (Old, Old-Enriched, Old-Wheel, Young). Data are mean ± SEM. **p≤0.01. A significant blade effect was found, $F(1, 28)=19.667$, $p<0.001$, as well as a blade by treatment interaction effect, $F(3, 28)=63.481$, $p<0.001$, $\eta^2=0.872$. A significant effect of treatment group was found in both the suprapyramidal, $F(3, 31)=36.242$, $p<0.001$, and infrapyramidal blades, Welch's $F(3, 14.407)=38.106$, $p<0.001$, with a significant difference found between the Young and the three remaining groups, $p<0.01$.