Neuronal Survival of the Fittest: The Importance of Aerobic Capacity in Exercise-Induced Neurogenesis and Cognition

by

Christina Maria Tognoni

Department of Psychology & Neuroscience
Duke University

Date: _________________________

Approved:

__________________________
Christina L. Williams, Supervisor

__________________________
Staci D. Bilbo

__________________________
Anne E. West

__________________________
Henry Yin

__________________________
Lee W. Jones

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Psychology & Neuroscience in the Graduate School of Duke University

2014
ABSTRACT

Neuronal Survival of the Fittest: The Importance of Aerobic Capacity in 
Exercise-Induced Neurogenesis and Cognition

by

Christina Maria Tognoni

Department of Psychology & Neuroscience 
Duke University

Date: _______________________

Approved:

____________________________
Christina L. Williams, Supervisor

____________________________
Staci D. Bilbo

____________________________
Anne E. West

____________________________
Henry Yin

____________________________
Lee W. Jones

An abstract of dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Psychology & Neuroscience in the Graduate School of Duke University  

2014
Abstract

It is commonly accepted that aerobic exercise increases hippocampal neurogenesis, learning and memory, as well as stress resiliency. However, human populations are widely variable in their inherent aerobic fitness as well as their capacity to show increased aerobic fitness following a period of regimented exercise. It is unclear whether these inherent or acquired components of aerobic fitness play a role in neurocognition. To isolate the potential role of inherent aerobic fitness, we exploited a rat model of high (HCR) and low (LCR) inherent aerobic capacity for running. At a baseline, HCR rats have two- to three-fold higher aerobic capacity than LCR rats. We found that HCR rats also had two- to three-fold more young neurons in the hippocampus than LCR rats as well as rats from the heterogeneous founder population.

We then asked whether this enhanced neurogenesis translates to enhanced hippocampal cognition, as is typically seen in exercise-trained animals. Compared to LCR rats, HCR rats performed with high accuracy on tasks designed to test neurogenesis-dependent pattern separation ability by examining investigatory behavior between very similar objects or locations. To investigate whether an aerobic response to exercise is required for exercise-induced changes in neurogenesis and cognition, we utilized a rat model of high (HRT) and low (LRT) aerobic response to treadmill training. At a baseline, HRT and LRT rats have comparable aerobic capacity as measured by a standard treadmill fit test, yet after a standardized training regimen, HRT but not LRT rats robustly increase their aerobic capacity for running. We found that sedentary LRT and HRT rats had equivalent levels of hippocampal neurogenesis, but only HRT rats had an elevation in the number of young neurons in the hippocampus following training, which was positively
correlated with accuracy on pattern separation tasks. Taken together, these data suggest that a significant elevation in aerobic capacity is necessary for exercise-induced hippocampal neurogenesis and hippocampal neurogenesis-dependent learning and memory. To investigate the potential for high aerobic capacity to be neuroprotective, doxorubicin chemotherapy was administered to LCR and HCR rats. While doxorubicin induces a progressive decrease in aerobic capacity as well as neurogenesis, HCR rats remain at higher levels on those measures compared to even saline-treated LCR rats. HCR and LCR rats that received exercise training throughout doxorubicin treatment demonstrated positive effects of exercise on aerobic capacity and neurogenesis, regardless of inherent aerobic capacity. Overall, these findings demonstrate that inherent and acquired components of aerobic fitness play a crucial role not only in the cardiorespiratory system but also the fitness of the brain.
# Table of Contents

Abstract ........................................................................................................................................ iv
List of Figures ............................................................................................................................... viii
Acknowledgements ....................................................................................................................... x

Introduction .................................................................................................................................. 1
  Effects of Exercise on the Brain ................................................................................................. 2
  Neurogenesis as an avenue for improving cognition ............................................................... 5
  Animal Models of Aerobic Fitness ............................................................................................. 9
  Experimental Strategy .............................................................................................................. 17

Chapter 1: A behavioral method for evaluating pattern separation ability that is sensitive to changes in hippocampal neurogenesis ................................................................. 20
  Methods .................................................................................................................................. 22
  Results ................................................................................................................................... 27
  Discussion ................................................................................................................................. 32

Chapter 2: Inherent and acquired aerobic fitness are determinants of enhanced hippocampal neurogenesis and pattern separation ability ........................................................ 36
  Methods .................................................................................................................................. 39
  Results ................................................................................................................................... 46
  Discussion ................................................................................................................................. 69

Chapter 3: Inherent and exercised-induced aerobic capacity are able to protect against chemotherapy-induced decline in cardiorespiratory and hippocampal fitness ................................ 78
  Methods .................................................................................................................................. 82
  Results: Experiment 1 ............................................................................................................. 86
  Results: Experiment 2 ............................................................................................................. 90
  Discussion ................................................................................................................................. 93

Conclusions .................................................................................................................................. 98

Appendix A: Voluntary Running Can Protect Against Whole-brain Irradiation-induced Neurocognitive Deficits Under Severe Immunodeficiency .................................................. 99
  Abstract ................................................................................................................................. 99
List of Figures

Figure 1.1: Object recognition task designed to test investigatory pattern separation ability. ................................................................. 24

Figure 1.2: Parametric object recognition task demonstrated a progressive decline in pattern separation ability following WBI. ........................................................................ 28

Figure 1.3: Object placement task confirmed memory impairment 7 months post-WBI. 29

Figure 1.4: Fear conditioning deficits 7 months post-WBI ............................................. 31

Figure 1.5: WBI induced a large reduction in hippocampal neurogenesis that was evident at 7 months post-WBI ............................................................. 32

Table 2.1: Primers for RT-PCR ........................................................................ 45

Figure 2.1: Rats selectively bred for high aerobic capacity have a higher rate of neuronal survival in the hippocampus compared to low aerobic capacity rats ..................................... 47

Figure 2.2: Triple-label fluorescent immunohistochemistry for NeuN, GFAP, and BrdU. ........................................................................................................................................... 49

Figure 2.3: Selection for high aerobic capacity conferred a phenotype of high levels of neurogenesis compared to the heterogeneous founder population ............................................ 51

Figure 2.4: Gene expression for potential neurogenic factors in FND, LCR, and HCR rats ........................................................................................................................................... 53

Figure 2.5: Characterization of neurogenesis in the SVZ-OB pathway in rats selected for high and low aerobic capacity .................................................................................................................... 55

Figure 2.6: Rats selectively bred for high aerobic capacity show enhanced performance on neurogenesis-dependent pattern separation tasks ............................................................................. 58

Figure 2.7: HCR, but not LCR, rats generalized conditioned fear and made stronger US-CS associations ........................................................................................................................................ 60

Figure 2.8: Only rats that show a high aerobic response to training show an increase in hippocampal neurogenesis ........................................................................................................................................ 62

Figure 2.9: Rats selectively bred for high, but not low, response to training show enhanced pattern separation ability following training .................................................................................................................... 64

Figure 2.10: Standard object recognition for rats bred for inherent and acquired differences in aerobic capacity .................................................................................................................... 65
Figure 2.11: Activity box data for rats bred for inherent and acquired differences in aerobic capacity ................................................................. 66

Figure 2.12: Novel social interaction in rats bred for inherent and acquired differences in aerobic capacity ................................................................. 68

Figure 3.1: Inherent high aerobic capacity protects rats from chemotherapy-induced decline in neurogenesis................................................................. 87

Figure 3.2: HCR rats have fewer quiescent microglia than LCR rats regardless of doxorubicin treatment .................................................................... 89

Figure 3.3: Training provides protection against chemotherapy in HCR but not LCR rats ...................................................................................... 91

Figure 3.4: Differential CORT responses to an aerobic fitness test......................... 93

Figure A.1. Experimental timeline...................................................................... 104

Figure A.2. Running reduced WBI-induced learning and memory deficits............ 112

Figure A.3. Running increased the survival of young neurons, but not cell proliferation, in the hippocampus two months after WBI............................................. 115

Figure A.4. Irradiation and voluntary running alter protein expression in the hippocampus two months after WBI.............................................................. 116
Acknowledgements

My graduate adviser, Christina Williams, and our collaborator, Lee Jones, who have provided invaluable mentorship and enthusiasm for my work on these projects.

The other members of my dissertation committee, Staci Bilbo, Anne West, and Henry Yin for their comments and questions that have provided direction and perspective.

Fellow graduate students, Ralph Michael Peace, Michael Lacagnina, and Lauren Williamson for their technical assistance and collaboration.

The laboratories of Staci Bilbo, Anne West, and Christine Drea for their resources, equipment, and guidance.

The Koch & Britton Lab for the selective breeding of rats used in these experiments.

Aynara Chavez Wulsin for her technical management and help.

Duke undergraduate students who contributed to the work completed throughout my graduate career: Rosalie Yan, Pam Bhullar, Emma Babb, Christopher Dermarkarian, Junmi Saikia, Kelly Andrejko, Jeanette Du, Shane Loomis, Caroline Tybout, and Naveen Nath.

Duke Mechanisms of Behavior students who have assisted me on projects conducted during the summer: Lauren Agoubi, Jessica Zhou, James Flannery, and Sara Maurer.

My good friends, Young May Cha, Carlos Mariscal, Erica Rodriguez, Shelley Alonso-Marsden, Cavin Ward-Caviness, Marek Łaska, Jessica Bolton, Bon Mi Gu, and other friends from the Duke community.

Funding and support from the National Science Foundation through the Graduate Research Fellowships Program (NSF-GRFP), Duke Conference Travel Award Funding, Duke Graduate School, and the department of Psychology & Neuroscience.
Introduction

It is well-known that aerobic exercise improves cognition and brain function in addition to physical fitness. However, exercise regimens can vary in intensity, duration, frequency, consistency, and type, and can have a range of effects on the cardiorespiratory system and the brain. Most exercise paradigms used experimentally are thought to strengthen cardiorespiratory fitness (CRF) by increasing aerobic capacity, or the body's ability to use oxygen (Gaesser and Rich, 1984). However, the majority of studies of the effects of exercise on the brain do not measure changes in aerobic capacity (e.g., via direct VO$_2$ max measurements or estimations using a fitness test, such as on a treadmill or cycle ergometer). Human populations have significant variability in both inherent CRF as well as in the capacity to show increased CRF following a period of regimented exercise (Bouchard et al., 1998, Bouchard et al., 1999, Bouchard et al., 2000). Despite these large differences in human CRF, research has not yet addressed the issue of whether the cardiorespiratory fitness aspect of exercise is a key component of neurocognition.

The main focus of this work is to elucidate how large differences in aerobic fitness affect the birth and survival of new neurons in the adult brain, accuracy on learning and memory tasks, and protection from a neurological assault. Whether or not these effects of aerobic fitness are specific to the hippocampus or another neurogenic brain region will also be determined, as well as the potential brain changes that may lead to these differences. Here, I will summarize the current literature on the effects of exercise on the brain, discuss the potential role of aerobic fitness in the benefits of exercise, and outline a strategy to differentiate components of aerobic fitness using selectively-bred rat models of inherent and acquired aerobic traits. These selectively-bred rats also give us the
opportunity to analyze baseline differences in aerobic fitness with or without the influence of exercise.

Effects of Exercise on the Brain

Exercise has been shown to improve cognitive function in both humans (Kramer et al., 2006) and rodents (Van Praag et al., 2005), enhance stress resiliency (Schoenfeld and Gould, 2013), and potentially provide protection against neuropathologies such as Alzheimer's disease or mild cognitive impairment (Foster et al., 2011). Studies on the effects of exercise on neurocognitive function have generally focused on aerobic exercise, whether that be in the form of low-intensity physical activity (e.g., daily walking or jogging in humans or voluntary wheel running in rodents) or a high-intensity training aimed at increasing endurance (e.g., interval training on a treadmill). Regardless of regimen, the positive effects of aerobic exercise on the brain seem to be concentrated in the hippocampus – a region of the forebrain with important roles in learning and memory as well as stress regulation – which experiences exercise-induced increases in synapses, dendritic branching, dendritic spines, growth factors, growth of new blood vessels as well as the proliferation and survival of new neurons, or neurogenesis (Voss et al., 2011). Because aerobic exercise causes a significant upregulation of neurogenesis in the dentate gyrus (DG) of the hippocampus, cellular and molecular mechanisms underlying these changes have become a major area of investigation.

A wide-range of factors (e.g., growth, hormonal, immune, neurotransmitter, etc.) has been implicated as being necessary for exercise's effects in the hippocampus. Vascular endothelial growth factor (VEGF), insulin growth factor-1 (IGF1), and, to an arguably less consistent extent, brain-derived neurotrophic factor (BDNF) have all been reported to be mediators of the effects of exercise on neurogenesis, spatial learning, and anxiety-like behaviors (Trejo et al., 2001, Fabel et al., 2003, Vaynman et al., 2004, Ding
et al., 2006, Cotman et al., 2007, Trejo et al., 2008). These growth factors can increase proliferation of neural stem cells as well as the proportion of newly born cells that differentiate and mature into neurons. Immune-associated mechanisms have also been implicated in neurogenesis: the use of immunodeficient mice has revealed that CNS-specific T cells are necessary for enrichment-induced increases in hippocampal neurogenesis, spatial learning and memory on the Morris water maze, and for the expression of BDNF in the DG (Ziv et al., 2006). CNS-specific serotonin has also been shown to be necessary for exercise-induced neurogenesis, particularly the rapid increase of cell proliferation (Klempin et al., 2013). In contrast, the local production of dihydrotestosterone (DHT), which binds to androgen receptors in the hippocampus, is necessary for exercise-induced enhancement in the survival, but not proliferation, of newborn neurons in rats (Okamoto et al., 2012). Treadmill running-induced down-regulation of glucocorticoid signaling via the mineralocorticoid receptor has been shown to induce neurogenesis by enhancing the differentiation of newborn cells into neurons (Chang et al., 2008). Recently, voluntary wheel running has been shown to improve anxiety regulation by increasing the stress-induced activation (but not number) of local GABAergic interneurons in the DG of the ventral hippocampus, which results in a lower percentage of newborn neurons that respond to a stressor in mice (Schoenfeld et al., 2013). Like in the embryonic nervous system, activated GABA-A receptors depolarize young DG neurons due to a high intracellular concentration of chloride ions and as these neurons mature, migrate, and integrate into the GCL, GABA becomes an inhibitory signal. Therefore, young neurons show increased excitability and are thought to modulate the activity of GABAergic interneurons and neighboring mature granular neurons in the dentate gyrus (Toni and Sultan, 2011). These observations are consistent with the view that GABA enables neural progenitors to sense neuronal activity and
regulate the rate of their development (Ge et al., 2007). In all, there are overlapping yet different factors that have been shown to be responsible for different stages of exercise-induced neurogenesis. One reason there seem to be many mechanisms for exercise-induced effects on neurogenesis could be because exercise is a complex behavior that has been experimentally manipulated in several ways and as such, has not been well-defined.

Adult neurogenesis also occurs in the subventricular zone (SVZ) of the lateral ventricles in the forebrain. The cells that proliferate in the SVZ migrate through the rostral migratory stream (RMS), where most differentiate into neurons or apoptose. The remaining young neuroblasts mature into interneurons and establish connections within the olfactory bulb (OB). Whether exercise influences SVZ-OB neurogenesis is less clear than exercise effects on neurogenesis in the DG. For example, voluntary wheel running in mice has been shown to stimulate the proliferation and survival of cells in the SGZ of the hippocampus, but neither cell proliferation in the SVZ nor the survival of new neurons in the olfactory bulb appear to be altered (Brown et al., 2003). Another study found that an enriched environment that includes a running wheel, but not voluntary running alone, is able to enhance proliferation in the SVZ following stroke-induced lesions in rats (Komitova et al., 2005). These data suggest that exercise in combination with other enriching conditions may create a more potent – or less stressful – stimuli for SVZ proliferation. More recent studies report a positive association between exercise and olfactory neurogenesis, particularly cell proliferation in the SVZ. Rats deprived of exercise by the hindlimb suspension model (in which the hind end of rats is elevated with a flexible string, but rats are free to move, drink, and eat with their forelimbs) have suppressed proliferation in the SVZ, suggesting that exercise experience is important for the rate of cell birth in the SVZ (Yasuhara et al., 2007). Interestingly, in a rat model of Parkinson's disease in which SVZ proliferation is low, voluntary wheel running rescues
SVZ neurogenesis (Tajiri et al., 2010). While there are discrepancies in the reported magnitudes of the effect, exercise seems to have the potential to enhance SVZ-OB neurogenesis, or at least rescue neurogenesis after a neurological disease or assault.

**Neurogenesis as an avenue for improving cognition**

The function of the hippocampus predominantly has been defined by its roles in learning, memory, stress, and depression, which are also the main behaviors that exercise seems to modify. While the addition of new neurons to this region in adulthood has been implicated all of these hippocampal functions, the precise role of neurogenesis is under current investigation. It is plausible that enhancing neurogenesis could be the main conduit by which exercise exerts its benefits on a number of cognitive behaviors.

The hippocampus has crucial roles particularly in reducing interference from overlapping, similar events (e.g., parking a car in different spaces from day to day in the same lot) and allowing for accurate integration of new information. To perform these functions, the hippocampal circuit undergoes neural computations to transform the representation of information. The DG subfield of the hippocampus has, in rats, five to ten-fold more principal neurons than layer II of the entorhinal cortex (EC) from which it receives most of its input (Amaral et al., 1990). Lower activity levels of these dense DG cells results in sparse coding, such that highly similar input is able to be separated into distinct representations, a process known as pattern separation [see review: (Yassa and Stark, 2011)]. It is thought that the CA3 either separates or completes these degraded or incomplete components based on previously stored representations via the strong, unidirectional input from the DG along the mossy fiber pathway, perforant path input directly from the EC, and recurrent collateral input from CA3 neurons. In other words, input is disassociated or associated with previous representations allowing for the encoding of new memories or recollection of previous memories, respectively. A high
capacity to separate patterns, therefore, allows for new representations that are similar but distinct from older representations.

While sparse coding appears to be an intrinsic property of mature granule cells in the DG, adult neurogenesis in this region creates an ongoing supply of young neurons that are more readily excitable than established neurons. Multipotent neural stem cells express the astroglia marker GFAP, and these type 1 progenitor cells proliferate along the subgranular zone (SGZ) and are thought to give rise to neuroblasts [see review: (Zhao et al., 2008)]. Neuroblasts, or type 2 neural progenitors, do not express GFAP but the stem cell transcription factor Sox2 and, in turn, divide and differentiate into young neurons that receive local GABAergic inputs that are excitatory due to a high concentration of intracellular chloride ions. These new neurons migrate along the DG, during which they undergo a transition from excitatory to inhibitory GABA and begin to receive excitatory glutamatergic input from the entorhinal cortex. The young neurons eventually integrate into the granular cell layer (GCL) of the DG where they develop processes to make synapses with CA3 neurons and become functionally integrated into hippocampal circuitry (Zhao et al., 2008). Factors at any stage (proliferation, differentiation, or maturation) could potentially affect the number of new neurons in the DG.

Newborn neurons in the DG are more readily excitable than established neurons, and a larger population of these cells could allow for enhanced formation of distinct memory traces (Becker, 2005). When hippocampal neurogenesis is upregulated in rodent studies, e.g., by environmental enrichment (Kempermann et al., 1998), prenatal choline supplementation (Glenn et al., 2007), or voluntary wheel running (van Praag et al., 1999), performance on memory tasks that rely on the hippocampus are improved. In contrast, experimentally-ablating DG neurogenesis with whole-brain irradiation (WBI), for example, decreases the performance of rodents on hippocampal memory tasks, such
as non-matching to sample (Winocur et al., 2006), the Morris water maze (Liu et al., 2010), and the Barnes maze (Wong-Goodrich et al., 2010). Long-term cognitive impairments and attention deficits are also observed in children (Krull et al., 2013) and adults (Anderson-Hanley et al., 2003) following WBI for cancer treatments, suggesting that loss of these dividing cells may also be critical to human cognitive function.

More recently, studies have revealed that neurogenesis in the DG plays an important role in the DG function of pattern separation, which includes both the neural and behavioral phenomenon of disambiguating similar patterns of information during learning tasks (Sahay et al., 2011b). For instance, when hippocampal neurogenesis is ablated with WBI, mice cannot discriminate between two closely-spaced food locations in a radial arm maze and cannot discern two closely spaced objects on a touch screen (Clelland et al., 2009). Voluntary running increases the overall number, but not proportion, of newborn cells that develop into DG neurons in 3-month-old mice, and this neurogenesis correlates positively with their ability to discriminate a rewarded stimuli and an adjacent, unrewarded stimuli presented on a touch screen (Creer et al., 2010). Aged, 22-month-old mice, on the other hand, have poor spatial discrimination and low basal cell genesis that does not significantly improve with running experience. Our laboratory has shown that voluntary running protects against the WBI-induced decline in neurogenesis and hippocampal flexibility for learning and remembering spatial locations on the Barnes maze in female mice (Wong-Goodrich et al., 2010). While not necessarily a pattern separation task, the enhanced flexibility in reversal learning and memory for a new spatial location that was different from a previously-learned location on the Barnes maze is consistent with the idea the exercise-induced enhanced neurogenesis improves the ability to disambiguate similar input. Unlike the proliferation-specific effects of exercise observed in Creer et al. (2010), we observed an
enhancement in both the number of newborn cells as well as the proportion that
differentiate into neurons (Wong-Goodrich et al., 2010). In order to demonstrate that an
increase in neurogenesis is sufficient to explain exercise's enhancement of pattern
separation, one study used a targeted genetic manipulation that promoted the survival of
adult-born granule cells in mice and showed that this gain in neurogenesis resulted in
improved performance on a contextual fear-discrimination learning task, which has been
proposed to require pattern separation in the DG-CA3 circuit (Sahay et al., 2011a).
Overall, considerable data support the contention that DG neurogenesis is both
necessary and sufficient to enhance hippocampal-dependent pattern separation ability.

Despite these gain or loss of function studies, the mechanism by which newborn
cells in the DG can improve pattern separation is not well understood. For instance, it is
unclear whether the younger, easily excitable neurons are responsible for enhanced
pattern separation ability or whether their maturation and integration ultimately lead to
an increase in neuronal density that improves pattern separation capacity in the DG. It is
possible that there are differences in consequences of short-term (e.g., immediate
changes in cell proliferation), long-term (e.g., progressive changes in differentiation,
neuronal survival, and integration), or lifetime (e.g., a genetic or developmental
modification) alterations in neurogenesis on pattern separation ability. Accordingly, it is
also possible that there are multiple types of pattern separation abilities (e.g., spatial
location separations, contextual fear discrimination) that are differentially affected by
the stages of neurogenesis that were altered or the context in which neurogenesis was
altered (e.g., inherent versus induced new neurons). The present studies will examine
some of these possibilities.

Unlike hippocampal neurogenesis where newborn cells mature into excitatory
glutamergic neurons, newborn SVZ cells that migrate to the OB incorporate as local
inhibitory GABAergic interneurons. The supply of these neurons in adulthood has also
been implicated in pattern separation ability, not for spatial patterns, but for olfactory
discrimination (Sahay et al., 2011b). However, the extent to which olfactory neurogenesis
occurs in humans, as well as its relevancy, has been debated (Sanai et al., 2011,
Bergmann et al., 2012, Macklis, 2012). It is possible that most humans do not live in a
rich olfactory environment and do not need to fine-tune their olfactory discrimination
ability; however, increased use (e.g., in chefs, winemakers, perfumers) may increase
neurogenesis, similar to what is observed rodents studies where olfactory enrichment
upregulates SVZ-OB neurogenesis (Rochefort et al., 2002). Olfactory neurogenesis also
appears to be activity-dependent, such that new born neurons will not establish
connections in the OB unless they are accompanied by neuronal activity (Cecchi et al.,
2001, Kelsch et al., 2009). Therefore, the function of a steady supply of neurons to the
olfactory bulb might be more relevant following a deficit or contingent on an increased
demand for processing complex olfactory information about the environment.

Together, the results of these studies support the view that in the hippocampus,
exercise-induced increases in neurogenesis appear to be directly responsible for
improving pattern separation ability and could potentially explain more generalized
improvements hippocampal-dependent learning and memory. Pattern separation ability,
which is also used in context-fear conditioning, could have implications for the
integration of stressful stimuli and contextual information. Aerobic exercise, therefore,
could be considered a mechanism for targeting hippocampal neurogenesis and
improving cognitive function across a broad range of behaviors.

Animal Models of Aerobic Fitness

Relatively novel rodent models of aerobic fitness have been used to specifically
address the role of variations in aerobic traits on a number of physiological measures,
and these models have only just begun to be used to investigate effects on the brain. Notably, selective breeding for treadmill running capacity from a heterogeneous N:NIH stock founder population (FND) has produced high-capacity runners (HCRs) and low-capacity runners (LCRs) that, by the 6th generation, differed by 170% in aerobic capacity in an untrained state (Koch and Britton, 2001). Aerobic capacity was measured using a standard fitness test designed to measure VO$_2$ max. A motorized treadmill was set at 15° slope and initial speed was set at 10 m/min with speed increasing 1 min/min every 2 min until an exhaustion criterion was met (defined by a rat refusing to run after being placed back on the treadmill 3 times).

Interestingly, selective breeding for high capacity running produced HCR rats that ran, on average, 839 ± 21 m compared to LCRs and FNDs that ran only 310 ± 8 m and 355 ± 20 m, respectively. With continued selection, the difference in aerobic capacity between HCR and LCR rats has increased to 347% by the 11th generation (Wisløff et al., 2005) and 414% by the 20th (Naples et al., 2010). The HCR/LCR model has been a valuable tool for investigating the potentially causal role of aerobic capacity in cardiac function (Hussain et al., 2001), cardiovascular risk factors (Wisløff et al., 2005), mitochondrial function (Koch and Britton, 2005), mitochondrial fatty-acid oxidation (Naples et al., 2010), cardiac protein glycosylation of mitochondrial proteins (Johnsen et al., 2013), insulin resistance (Noland et al., 2007, Haram et al., 2009), lipid and glucose metabolism (Bye et al., 2008), skeletal muscle response to antioxidant administration (Huettemann et al., 2013), and longevity (Koch et al., 2012). Inherent aerobic capacity, even in the absence of exercise training, is clearly a multifactorial trait that has implications for complex disease as well as normal biochemistry and physiology.

In addition to their physiology, HCR and LCR rats also differ in some aspects of their neurobiology, cognitive behavior, and stress responsivity. For instance, female HCR
and LCR rats appear to have differential stress responses to eight weeks of voluntary wheel running, with HCRs exhibiting decreased and LCRs exhibiting increased corticosterone compared to sedentary controls; HCRs also have more striatal dopaminergic activity (DA/DOPAC) than LCRs at a baseline, but not following running wheel experience (Waters et al., 2008). These differences in the regulation of endocrine and monoamine systems in HCR and LCR rats could influence a variety of behaviors, such as anxiety and motivation, which could modify their reactions to exercise, to being in a sedentary state, or to stress or novelty in the environment.

At a baseline, HCR rats have significantly lower levels of locomotion and rearing in a 60-min novel environment test (but no differences in grooming behavior) compared to LCR rats, suggestive of more anxiotypic behavior in HCR rats (Waters et al., 2010). Additionally, while HCR and LCR rats perform similarly on the elevated-plus maze (EPM) at a baseline, 24 hr after 1 h period of restraint stress, HCR rats display more anxiety-like behavior. Additionally, this combination of EPM exposure following restraint stress elicited a significant increase in plasma corticosterone in HCRs but not LCRs. Administration of dexamethasone, a potent glucocorticoid, resulted in a normal suppression of plasma corticosterone in all rats, suggesting that both HCR and LCR rats have similar negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis.

Another study found no locomotor activity differences in male HCR and LCR rats in a novel environment test (Burghardt et al., 2011). However, male HCR rats did show greater anxiotypic behavior in a light/dark box task as well as greater plasma corticosterone responses following the task. While HCR and LCR rats showed similar decreased exploration in response to cat odor, HCR rats showed greater contextual conditioning to cat odor. While Burghardt and colleagues (2011) did not find significant differences in mineralocorticoid or glucocorticoid receptor mRNA expression throughout
the DG, CA3, CA2, and CA1 subregions of the hippocampus, they did find that HCR rats had increased adrenal and decreased thymus weights as well as greater corticotropin-releasing hormone levels in the central nucleus of the amygdala. On the other hand, LCR rats displayed more immobility in a forced swim test compared to HCR rats, even following antidepressant treatment, which could suggest that LCR rats have more depressive-like behavior. It seems more likely, however, that the inherent differences in aerobic fitness of these rats confounds a forced swim task, which requires physical endurance along with motivation. Burghardt et al. (2011) interpret the overall finding of higher anxiotypic behavior in HCR rats as an advantageous strategy for coping with and assessing the risk of environmental novelty or potentially dangerous, salient signals, and attributes it to differences in stress regulation.

A few cognitive behaviors have also been evaluated in LCR and HCR rats. While no baseline differences have been found in trace-fear conditioning (a hippocampal-dependent task), HCR rats have been shown to be partially protected from a decline in freezing behavior to the context 3 days following surgery (aseptic trauma involving tibial fracture) (Su et al., 2012). Surgery induced this postoperative cognitive decline alongside an increase in hippocampal expression of pro-inflammatory cytokine IL-6, which is more exaggerated in LCR rats. Surgery also induced an increase in hippocampal M2 macrophages, but more-so in HCR rats, indicating an activation in macrophages to protect against neuroinflammation. Overall, HCR rats seem to be more responsive to stressful stimuli (e.g., from fear conditioning and surgery) and this responsivity may be neuroprotective. Further support of the finding of greater stress responsivity in HCR rats is the finding that HCR rats showed better passive-avoidance behavior than LCR rats, staying longer in a bright chamber and avoiding a dark chamber where they were previously shocked (Sarga et al., 2013).
Selective breeding for inherent high aerobic capacity seems to particularly enhance cognitive learning. HCR rats outperform LCR rats on an appetitively-motivated discrimination-reversal classical conditioning task as well as an alternating T-maze task (Wikgren et al., 2012). Differences between HCR and LCR rats were evident during reversal learning phases of these tasks, which require cognitive flexibility. Not surprisingly, HCR rats also demonstrated more agility than LCR rats on a Rotarod task, but both groups of rats improved across trials, indicating that inherent aerobic capacity does not affect motor learning. While overall these findings suggest that HCR rats have enhanced cognitive learning and memory compared to LCR rats, they do not rule out the possibility that differences in motor ability, appetitive motivation, and anxiety-like behavior could be the reason that HCRs and LCRs differ on these cognitive tasks that require movement, motivation for a food reward, or response to a stressor.

Activity-dependent neural factors have also been evaluated in the HCR/LCR model. While no strain differences in hippocampal BDNF mRNA have been reported, male HCR and LCR rats, like Sprague Dawley rats, show an elevation in BDNF mRNA in the CA1 following 3 weeks of voluntary wheel running compared to sedentary controls (Groves-Chapman et al., 2011). These results support findings that while BDNF is increased by exercise, BDNF expression levels are not related to total running distance accumulated across a 3 week period (which differ among HCR, LCR, and Sprague Dawley rats that voluntarily run 4726 ± 3220, 672±323, and 2293±3461 m/day, respectively) or to differences in inherent aerobic capacity. HCR and LCR rats do, however, show differential cFos mRNA activation in response to running (Foley et al., 2012). Following a fitness test for running to exhaustion, HCR rats showed greater cFos in the majority of brain regions studied, including the CA3 of the hippocampus, the raphe magnus nucleus (RMg) of the brain stem, the arcuate nucleus (Arc) and
paraventricular nucleus (PVN) of the hypothalamus. There were no HCR-LCR differences in cFos in the hippocampal CA1 region, and LCR rats had lower cFos in specifically the inferior olivary nucleus (OLV). When running was limited to the duration of the maximal aerobic capacity of LCR rats (15 min), HCR rats showed greater cFos in the CA1 and RMg but were not different from LCR rats in cFos in the CA3 and the majority of other regions studied. Taken together, these results could suggest that a single running experience fails to induce the hippocampal expression of activity-dependent genes (at least cFos) in LCR rats compared to HCR rats, but a long-term period of running increases activity-dependent genes (at least BDNF) similarly in LCR and HCR rats.

LCR and HCR rats also appear to have differences in antioxidant and oxidative DNA damage-repairing systems, which are mostly evident after training (Sarga et al., 2013). Following a 12-week-long individualized training regimen, HCR, but not LCR, rats had elevated oxidized proteins in the hippocampus as measured by an Oxiblot kit. While sedentary HCR rats compared to LCR rats had lower Ogg1, a DNA glycosylase that repairs 8-oxoguanine that result from reactive oxygen species (ROS), and similar levels of SIRT1, a NAD+-dependent deacetylase sirtuin that is a known regulator of mitochondrial biogenesis marker PGC-1α, training increased hippocampal expression of Ogg1 and SIRT1 only in HCR rats. Only trained LCR rats experience an increase in acetylated Ogg1 (AcOgg1) accompanied by a decrease in SIRT1, which was demonstrated to be a regulatory factor of Ogg1. These findings suggest high aerobic capacity is associated with enhanced oxidative DNA repair and metabolic function in the hippocampus.

One major unanswered question about the HCR/LCR rats is what genes might have been selected for during the breeding process that resulted in their drastic
differences in inherent aerobic capacity. A gene screening of the mRNA from the left ventricle of the heart of HCR and LCR rats found that out of 28,000 screened genes, 1,540 were differentially expressed between sedentary HCR and LCR rats (Bye et al., 2008). Many of these genes were associated with cardiac energy substrate, growth signaling, and cellular stress. Only one mRNA sequence was found to be significantly different between trained HCR and LCR rats, and it was of unknown name and function.

A major limitation of mRNA analysis is that it might not necessarily represent a difference in protein levels or intrinsic differences in the genome. DNA sequencing of HCR and LCR rats would need to be conducted in order to determine the genes that were selected from this breeding scheme and the extent to which differences in HCR and LCR rats are due to the genetic sequences. One study has conducted mathematical analysis to confirm that the trait of aerobic capacity selected in HCR and LCR rats is highly heritable (Ren et al., 2013). The authors conclude that heritability pattern indicates a strong genetic component and that HCR/LCR rats could serve as a valuable model of genetic evolution. However, it still remains possible that some, if not all, of the differences between HCR and LCR rats are due to epigenetic heritability. Regardless whether the heritability of aerobic capacity is genetic, epigenetic, or some combination, the HCR/LCR rat model provides a valuable tool for studying how large differences in aerobic capacity influence the body and brain.

A rat model of differences in acquired aerobic fitness has also been developed by selective breeding for high (HRT) verses low (LRT) response to training. At a baseline, HRT and LRT rats have comparable aerobic capacity as measured by a standard treadmill fitness test, yet after a standardized endurance training regimen, HRT rats significantly increase their aerobic capacity for running compared to LRT rats (Koch et al., 2013). These rats were also bred from an N:NIH stock founder population, which
respond to exercise training on average with a $140 \pm 15$ m gain in aerobic capacity ($\Delta \text{DIST}$). The training regimen occurred on a treadmill set at 15° slope and an initial speed of 10 m/min and duration of 20 min, increased speed 1 m/min every other session up to a maximum speed of 21 m/min, and increased duration 0.5 min each session to a maximum target of 31.5 min by session 24. Note that this regimen was standardized such that each rat experienced the same amount of exercise from day-to-day. After 15 generations of selection, HRT rats showed a $\Delta \text{DIST}$ of 223 ± 20 m on a fitness test given before and after training, while LRT rats had a $\Delta \text{DIST}$ of -65 ± 15 m. Following the training period, HRT and LRT rats differed in aerobic capacity by approximately 160%. Unlike the selection for the HCR/LCR rats that produced drastic, non-overlapping differences in inherent aerobic capacity, some overlap in $\Delta \text{DIST}$ of aerobic capacity remained in generation 15 of the HRT/LRT rats.

Research has just begun to characterize differences in the physiology of HRT and LRT rats. The entire transcriptome has been analyzed from the skeletal muscles of these rats, revealing approximately 800 response-to-training-regulated genes, with many related to proangiogenic and tissue developmental networks, such as RUNX1, PAX3, and SOX9 (Keller et al., 2011). This mRNA-level analysis was compared to several genomic DNA variants that associated with maximal aerobic capacity trainability in human subjects obtained from the HERITAGE Family Study (Bouchard et al., 1999), but these were not significantly different following a conservative Bonferroni adjustment. Another study found that aerobic exercise training of LRT rats causes pronounced metabolic dysfunction, which is characterized by insulin resistance and increased adiposity compared to exercise-trained LRT rats (Lessard et al., 2013). LRT rats also showed impaired exercise-induced angiogenesis in skeletal muscle. Mitochondrial capacity of muscle, on the other hand, was intact and increased similarly in LRT and HRT rats from
exercise training. This study also found that LRT rats appeared to have increased stress and inflammatory signaling and altered transforming growth factor-β (TGF-β) signaling, characterized by hyperphosphorylation of a novel exercise-regulated phosphorylation site on SMAD2 protein. These findings suggest that low-responders are actually disadvantaged or unchanged from exercise training, in contrast to a number of positive benefits seen in rats that are highly responsive to exercise training. Whether or not these differences in aerobic response to training affect the brain and behavior is investigated in the current body of work.

**Experimental Strategy**

The overall objective of these studies is to investigate whether components of aerobic fitness (i.e., inherent or acquired by exercise) are critical for improving hippocampal function. To manipulate these components, we used selectively-bred rodent models of the inherent aerobic capacity for running (HCR/LCR rats) and of the capacity to show an aerobic response to exercise training (HRT/LRT rats). These rat models produced from selective breeding act as powerful tools in examining the effects of marked differences in these components of aerobic fitness without having to rely on the natural variability within large populations. The present experiments are designed to test how these variations in fitness might influence hippocampal neurogenesis and pattern separation ability, and to compare these findings to what has previously been accepted about exercise-induced effects on the brain.

Described in the first chapter is a new method to evaluate pattern separation ability that appears to be neurogenesis-dependent. This procedure relies on rats' natural exploratory tendencies and thus, is a direct measure of cognitive ability that is not likely confounded by differences in aerobic fitness (e.g., weight, speed, muscle strength,
motivation for food). First, I examined pattern separation ability in whole-brain irradiated (single dose of 5 Gy) and sham irradiated female Sprague Dawley rats on 4 different cognitive tasks: a standard object placement task, a novel version of an object recognition task that varied the shape of objects made from plastic blocks to parametrically test pattern separation ability, a standard object placement task, and context fear conditioning. Loss of neurogenesis reduced rats’ ability to separate subtle changes in the shape or placement of objects suggesting that irradiated rats over-completed patterns. In our fear conditioning task, loss of hippocampal neurogenesis resulted in an inability to associate a context with a fear-inducing event. These data support the role of hippocampal neurogenesis in not only several types of pattern separation but also in the integration of contextual and aversive information.

Second, I addressed whether enhanced aerobic capacity, independent of exercise, is sufficient to result in high levels of hippocampal neurogenesis and protect against an impairment in neurogenesis. Hippocampal neurogenesis and pattern separation ability were examined in rats with of high (HCR) and low (LCR) intrinsic aerobic capacity for running. At a baseline, HCR rats have 2- to 3-fold higher aerobic capacity than LCR rats. We found that HCR rats also had 2-3 times more young neurons in the dentate gyrus than LCR rats as well as rats from the founder population (N:NIH heterogeneous stock rats). Additionally, HCR rats, like trained HRT rats, performed with high accuracy on pattern separation tasks compared to LCR rats. To investigate the role of aerobic capacity in exercise-induced changes in neurogenesis and neurocognition, we then utilized a rat model of high (HRT) and low (LRT) intrinsic aerobic response to treadmill training. At a baseline, HRT and LRT rats have comparable aerobic capacity, yet after a standardized training regimen that occurs 3 days/week for 8 weeks, HRT but not LRT rats increase their aerobic capacity for running. Congruent with this phenotype, I found
that only HRT rats have an elevation in the number of young neurons in the hippocampus following training, which was positively correlated with accuracy on pattern separation tasks. This result suggests that an increase in aerobic capacity is required for exercise-induced increases in hippocampal neurogenesis and cognition. Taken together, these data suggest that an enhancement in aerobic capacity is critical for an elevation in hippocampal neurogenesis and pattern separation ability.

The aim of the third chapter was to investigate the potential for high aerobic capacity to be neuroprotective from an oxidative assault, as well as the potential for exercise training to rescue a decrease in aerobic capacity and neurogenesis. Doxorubicin (dox), which does not cross the blood brain barrier significantly, was administered to HCR and LCR rats to investigate whether intrinsic aerobic capacity may protect against the neural and cognitive consequences of a peripheral assault on the cardiovascular system. While dox treatment decreased the number of young neurons in the hippocampus of both HCR and LCR rats, this decline in new neurons was significantly larger in the LCR rats. These data suggest that intrinsic high aerobic capacity provides protection against dox-induced impairments in new neuron survival. Analysis of the brains of HCR and LCR rats that received treadmill training demonstrated positive effects of exercise on aerobic capacity and neurogenesis, regardless of inherent aerobic capacity.

Taken together, these findings demonstrate that inherent and acquired components of aerobic fitness play a crucial role not only in the cardiorespiratory system, but also the brain. These findings could have implications for the recommendation of physical exercise as a cognitive therapy in human populations, which are widely variable in aerobic fitness.
Chapter 1: A behavioral method for evaluating pattern separation ability that is sensitive to changes in hippocampal neurogenesis

The function of hippocampal neurogenesis has been most recently attributed to pattern separation, which describes the cognitive and neural phenomenon of separating highly similar input into distinct representations (Yassa and Stark, 2011). However, current protocols for testing neurogenesis-dependent pattern separation ability require food deprivation, foot shocks, or swimming, which might not be appropriate for examining differences in pattern separation ability in rodents that differ in motivation for food, stress or pain responsivity, or swimming ability, for instance. Our objective was to develop a method that could test the level of pattern separation ability in rats that differ in physical fitness (e.g., weight, speed, muscle tone, aerobic capacity) that could also more broadly be acceptable to test pattern separation in a variety of rodent models. A protocol to parametrically test different levels of neurogenesis-dependent pattern separation ability that would not be significantly confounded by factors other than cognitive ability would be valuable for current research on hippocampal neurogenesis and function.

Decreases, such as from focal X-ray irradiation (Clelland et al., 2009), or increases, such as from wheel running (Creer et al., 2010) or genetic manipulation (Clelland et al., 2009), in hippocampal neurogenesis have been correlated with spatial pattern separation ability on a radial arm maze task and an operant task using touch screens. Contextual fear conditioning has also been used to test pattern separation in X-ray irradiated mice, control mice, and mice with genetically-increased neurogenesis, which all show identical high levels of freezing to an original context where footshocks had previously occurred, but differ respectively in discrimination of the original context.
and a distinct, yet similar context after repeated exposures (Sahay et al., 2011a). Overall, these tasks require food deprivation and food reward-motivated behavior, or a fear response to pain, and rely on mice being similar across these measures despite the manipulation of neurogenesis.

In rats, knocking-down hippocampal neurogenesis results in deficits on the Morris water maze and standard object recognition at a retention delay of 3 hr, but not 1 min or 4 wks (Jessberger et al., 2009). Aged rats, which have low levels of neurogenesis, have also been shown to have deficits on a standard object recognition task following a 2 h delay (Burke et al., 2010). While these long delays make the standard object recognition task more challenging and might place more weight on hippocampal function, the standard object recognition task is not always considered hippocampal-dependent. Additionally, standard object recognition arguably does not involve the separation of patterns. The standard object placement task, on the other hand, is known to be a hippocampal-dependent task due to its reliance on spatial location memory and potentially could be useful for evaluating differences in neurogenesis levels by varying the length of delay.

In humans, a pattern separation task can involve presenting visual stimuli that is identical, similar, or different from previous images. However, because it is typically unethical to examine neurogenesis in humans, these tasks can only, at best, relate visual accuracy to DG/CA3 fMRI activity (Yassa and Stark, 2011). We sought to develop a pattern separation task for rodents similar to testing human memory for previously-seen images and based off a standard object recognition paradigm. This task would 1) take advantage of rodents’ intrinsic motivation to investigate novel objects, 2) parametrically test degrees of pattern separation ability for dissimilar versus similar pairs of objects, and 3) be sensitive to increases or decreases in neurogenesis.
To do this, we designed pairs of objects with plastic building blocks that were classified as dissimilar (a geometric configuration) or similar (a featural configuration) that could be used create a less difficult or more difficult object recognition test, respectively. We also utilized a pair of completely distinct objects (e.g., a soda can vs. a mug) for a standard object recognition task to examine memory independent on hippocampal neurogenesis. We used these parametrically-different tasks to examine pattern separation ability in X-ray irradiated (a single dose of 5 Gy) and sham-irradiated rats. This low dose of whole-brain irradiation (WBI) is known to decrease hippocampal neurogenesis and cause progressive spatial learning and memory deficits over the next 1-2 months, while voluntary exercise during that period prevents this decline in memory and enhances hippocampal neurogenesis (Wong-Goodrich et al., 2010). We also examined performance on object placement and contextual fear conditioning to compare neurogenesis-dependent pattern separation ability across multiple tasks.

**Methods**

**Animals**

Twelve, 5-week-old female CD Sprague Dawley Rats (Charles River, Raleigh) were housed two per cage in ventilated shoebox cages with food and water *ad libitum*. The room was kept on a reversed light cycle with lights off from 09:00-21:00. At 4 months-of-age, all rats were anesthetized with 60 mg/kg ketamine and 5 mg/kg xylazine and given a single dose of 5 Gy cranial X-ray irradiation (IRR, n = 6) or sham irradiation (SHAM, n = 6). After recovery of 7 days, behavioral training began.

**Object Recognition**

All rats were given 2 consecutive days of a 5 minute acclimation to a 76 cm L x 30 cm W x 30 cm H open field with clear, Plexiglas walls. The open field was placed evenly
on top of a piece of white paper with a 12 cm x 10 cm grid and set on a table elevated 76 cm off the ground. One black LEGO (Billund, Denmark) platform was taped to the field floor, centered 12 cm from each short wall of the rectangular field. A circular curtain surrounded the apparatus to obscure features of the testing room. On each test day, rats were given another 5 minute acclimation minute followed by a 5 minute train trial, where two identical objects were placed on the platforms, followed by a 5 minute test trial, where one of the objects was changed (Fig. 1.1A). Inter-trial intervals were 3 hours, a duration previously demonstrated to reveal deficits by a decline in neurogenesis in rats during a standard object recognition task (Jessberger et al., 2009). The objects either underwent a standard change (e.g., a soda can versus a coffee mug), a geometric change (e.g., the LEGO design was built upside-down), or a featural change (e.g., gem-like pieces were added to the LEGO object) to test easier (standard), difficult (geometric), or most difficult (featural) levels of discrimination, respectively. Rats were given multiple rounds of testing for the geometric configuration that occurred at 1 week, 3 weeks, and 2 months following irradiation. To avoid bias from saliency of particular objects, multiple object sets were used, and these object sets were randomized across subjects throughout a test day. Rats were also given a different object set for each testing day such that they were not re-exposed to the same objects. Examples of three pairs of the geometric configurations are provided (Fig. 1.1B). The detailed configuration and standard change were only included during the last rounds of testing, which also included additional testing days that used a 1 minute inter-trial interval. Investigatory behavior (i.e., nose facing object within 2 cm or front paws touching object) during the first 3 minutes of each video-recorded test trial was timed by experimenters who were blind to the rat’s experimental condition. We found that 3 minutes was an ideal duration to time as by 1
minute, investigatory time tended to be too low to reveal significant differences and by 5 minutes, eventual habituation to the objects diluted any effect.

**Figure 1.1: Object recognition task designed to test investigatory pattern separation ability.** A) Habituation, trial, test for an object recognition test designed to test discrimination of a familiar object (bottom left triangle) from a similar object (bottom right triangle) that had undergone a geometric configuration. B) Examples of three object pairs for this task.

**Object Placement**

Following object recognition testing, an additional black plastic platform was taped to each side of the rectangular field, allowing for more horizontal area that an object could be fastened. Additionally, black material was placed on the outside of the field along one long and one short Plexiglas wall to create geometry in the environment.
This would allow rats to not only rely on the distance between two objects, but also their relation to the geometry of the walls, to recognize if an object had been moved. Rats were given a 3 minute acclimation period followed by a 3 minute train trial, where two identical LEGO objects were placed on the platforms, followed by a 3 minute test trial, where one of the objects had been moved. The objects were placed on either the inner or outer edge of the black platform and then moved by 15 cm to the outer or inner edge, respectively. Position combinations were randomized for each rat tested. Each rat experienced two object placement tests: one with a 1-minute and one with a 3-hour inter-trial interval. Investigatory behavior (i.e., nose facing object within 2.5 cm or front paws touching object) during the 1st minute of each video-recorded test trial was timed by experimenters, blind to the rat’s experimental condition.

**Context fear conditioning**

On the day fear conditioning (FC) occurred, rats were carried with their cagemate in a black bucket with a lid to the novel FC room. Rats were placed in FC boxes (in dim light, with QTB used as disinfectant and odor) for 2 minutes, followed by a 15 second-long tone, which was immediately proceeded by a 2 second-long 1.3 mA foot shock. Rats were then removed from the boxes, placed in the bucket without the lid, and returned to their home cage. After a 48-hour delay, rats were carried back to the FC room in the same bucket and were placed in the same FC boxes for 6 minutes (same context, but no tone nor shock occurred) as a “same context” test. Rats were immediately removed from the boxes, placed in the bucket without the lid, and returned to their home cage. After 3 hours, rats returned to the FC room in a novel red bucket. Rats were placed in novel boxes (that differed significantly from the original FC boxes: plastic floor rather than metal bars, triangular shape, patterned walls, in red light, and with ethanol used as disinfectant and odor) for 3 minutes (with no tone nor shock) as a “different context” test.
followed by 3 minutes of a constant tone with no shock as a “tone” test. Rats were immediately removed from the boxes, placed in the bucket without the lid, and returned to their home cage. Freezing was scored by 2 experimenters, one who noted if freezing was occurring every 5th second and one who noted if freezing was occurring every 10th second throughout the session: 1 for freezing, 0 for no freezing. Freezing behavior was categorized as a rat being virtually still with no sniffing or grooming behavior.

**Histology**

Upon sacrifice 7 months post-WBI, right hemispheres were post-fixed in 4% PFA and the hippocampus, prefrontal cortex, and olfactory bulbs were dissected from the left hemispheres, flash frozen in isopentanol on dry ice, and stored at −80º. Right hemispheres were sliced at 60 µm in order to perform immunohistochemistry for doublecortin (DCX) to evaluate levels of young neurons in the hippocampus as previously described (Van der Borght et al., 2009). Sections containing immunostained DCX+ cells were quantified using Stereo Investigator (MBF Bioscience, Williston, VT). Contours were drawn around the region that encompassed the dorsal and ventral blades of the DG, including the granule cell layer and subgranular zone using a 40x objective lens on a Nikon light microscope. Volume estimates were generated using the optical fractionator and according to Cavalleri’s principle (Mouton, 2002). There were no significant differences in DG volume obtained from these contours across experimental conditions. We used a modified fractionator principle to move exhaustively throughout each region, using an optical dissector height of 20 µm with a 2 µm guard zone to avoid over-sampling between brain sections. To represent the extent of the dorsal DG, 5 sections (each separated by a step of 300 µm) per rat were quantified. Data are represented as the number of cells per dorsal DG in one hemisphere.
Results

**WBI produced a progressive decline in pattern separation of similar objects**

Investigatory time spent with the familiar and novel object was analyzed for each of the object recognition tasks that used a geometric change. As expected, at 1 week post-WBI, sham (paired t-test, $p < 0.05$) and irradiated rats (irr, $p < 0.05$) similarly were able to recognize a change in the geometric shape of an object following a 3-hour delay (Fig. 1.2A). By 3 weeks post-WBI, irradiated (NS), but not sham ($p < 0.01$), rats were impaired on the same task. Two months (9 weeks) post-WBI, rats were again evaluated on an object recognition test for a geometric change, but this time following a 1 minute delay (Fig. 1.2A). Sham rats showed recognition of the novel object ($p < 0.05$), while irradiated rats were again impaired at the task (NS).

To test rats on a potentially more difficult pattern separation task, sham and irr rats were exposed to pairs of objects that varied by a featural change (Fig. 1.2B). At 3 weeks post-WBI and using a 3 hr delay, neither sham (NS) nor irr (NS) rats were able to recognize this change. At 9 weeks post-WBI but using a 1 min delay, however, sham ($p < 0.05$) but not irr rats showed accuracy on this task. Taken together, these data demonstrate that permutations in the degree of change in objects as well as the inter-trial interval can parametrically evaluate levels of pattern separation ability.

As a control for hippocampal-independent ability, rats were also tested on object discrimination for completely different objects. Both irradiated ($p < 0.05$) and sham ($p < 0.05$) rats displayed significant recognition of the novel object (Fig. 1.1C).
Figure 1.2: Parametric object recognition task demonstrated a progressive decline in pattern separation ability following WBI. A) Object recognition of a geometric configuration (e.g., up- vs. down-facing pyramid) prior to WBI and 1, 3, and 9 weeks post-WBI. Mean time spent with familiar and novel object from a 3 minute test at a 3 hour or 1 min delay. *p < 0.05. B) Object recognition of a featural configuration (e.g., with vs. without orange pieces) 3 and 9 weeks post-WBI. *p < 0.05. C) Object recognition for distinct objects (e.g., mug vs. can) (hippocampal-independent task). *p < 0.05.

WBI introduced a long-term deficit in pattern separation of similar spatial locations

Seven months post-WBI, rats were tested on a standard object placement task (Fig. 1.3A). Sham (p < 0.05) but not irradiated (NS) rats were able to recognize that an object had moved by 15 cm following a 1 minute delay (Fig. 1.3B). Following a 3 hour
delay, however, neither sham nor irradiated rats significantly spent more time with the novel placement of an object (Fig. 1.3C). These data parallel the differences in pattern separation ability observed in sham and irradiated rats on the geometric and featural object recognition tasks using a 3 hour delay.

**Figure 1.3: Object placement task confirmed memory impairment 7 months post-WBI.** A) Three-trial object placement protocol in which rats were habituated to a plexiglass container, returned to their home cage for a delay, trained with two identical objects (e.g., plus-shaped objects), returned to their home cage for another delay, and then tested for investigation of one of the objects that had moved either to the left or right. Time spent with the familiar or novel placement following a 1 min (B) or 3 hour (C) inter-trial delay. *p < 0.05.

**WBI resulted in impaired formation of context-fear associations**

To exam the extent to which whole-brain irradiation impacts contextual fear conditioning and the potential for overgeneralization of fear to a different context, rats were also evaluated on a standard context fear conditioning task 7 months post-WBI.
(Fig. 1.4A). In the original context where the a fear conditioning event occurred forty-eight hours earlier, sham rats displayed high levels of freezing (46 ± 6.8%), while irradiated rats showed significantly less levels of freezing (13 ± 5.7%, \( p < 0.01 \)) (Fig. 1.4B). When rats were tested in a different context that was in the same room as the fear conditioning event, both sham (7 ± 3.3%), and irradiated (9 ± 7.6%) rats similarly showed minimal levels of freezing. During the amygdala-dependent tone test, both sham (80 ± 7.4%) and irradiated (50 ± 13.5%) rats showed high levels of freezing; however, irradiated rats showed some deficit in their freezing response to the tone (one-tailed Student’s t-test, \( p < 0.05 \))
Figure 1.4: Fear conditioning deficits 7 months post-WBI. A) Fear-conditioning protocol in which freezing behavior was scored during a 6 minute test in the conditioned context (hippocampus dependent), a 3 minute test in a completely different context (generalization), and a 3 minute tone test in the different context (amygdala-dependent) following a 48-hour delay from the training experience. B) Percent freezing to the original context, different context, and tone. *p < 0.01, ^p < 0.05.

WBI targeted hippocampal neurogenesis

As expected, irradiated rats expressed fewer DCX+ young neurons (p < 0.01) in the dentate gyrus of the hippocampus at the time of sacrifice, 7 months post-WBI, compared to sham rats (Fig. 1.5).
Figure 1.5: WBI induced a large reduction in hippocampal neurogenesis that was evident at 7 months post-WBI. A) Compared to Sham rats, Irr rats had significantly fewer DCX+ young neurons in the dentate gyrus (DG) of the hippocampus 7 months post-WBI. *p < 0.01. B) Representative photo-micrographs of DCX+ neurons (stained dark gray) in the DG of a Sham and Irr rat.

Discussion

The use of a neurogenesis-dependent pattern separation task that takes advantage of the intrinsic motivation of rodents to investigate novel objects is ideal for testing rats that might differ in other attributes, such as physical fitness, sex, weight, or food reward motivation. Here, we designed a novel version of an object recognition task that involves parametrically varying in the degree to which an object is changed or the length of the delay between training and testing trials. Rats irradiated to diminish
hippocampal neurogenesis were not able to recognize that a small featural change in a LEGO object had occurred following a 1 min delay, while sham rats spent more time exploring the altered object. Neither irradiated or sham rats were able to recognize a featural change following a 3 hr delay. Sham but not irradiated rats were able to recognize if a LEGO object had underwent a more obvious geometric inversion at a 1 min or 3 hr delay. In contrast, both sham and irradiated rats had no difficulty noticing and spending more time exploring a completely different non-LEGO block object, following 1 min or 3 hr delay (presumably a hippocampal-independent task). The use of a range of dissimilar to similar pairings of objects in an object recognition task provides a unique tool for examining the cognitive consequences of decreases in hippocampal neurogenesis and has the potential to be sensitive to increases in neurogenesis levels. Additionally, the use of LEGO objects allows for the potential of this task to examine different types of visual and tactile pattern separation abilities, such as the separation of featural versus geometric cues.

To confirm the findings from the object recognition task, we also tested rats on a spatial pattern separation task. We demonstrated that irradiated rats were impaired in their ability to recognize a small change in placement of an object after a 1 min delay, while sham rats had no difficulty noticing and exploring the moved object. The use of a 3 hr delay revealed that neither sham or irradiated rats were able to recognize an object that had moved. Particular through the use of different delay lengths, this object placement task can also be valuable for examining differences in hippocampal neurogenesis.

We also examined context pattern separation using a fear conditioning task. For training, rats were placed in a Plexiglas box, and following the playing of a tone, a foot-shock was administered. Surprisingly, when rats were placed back into the conditioned
context 48 hrs later, irradiated rats showed significantly less freezing than sham rats. When placed in a different context (a box with a different shape and odor) to test generalization of conditioned fear, both sham and irradiated rats showed minimal freezing, but both showed similar high levels of freezing when the tone occurred (hippocampal-independent learning). Loss of neurogenesis reduced rats’ ability to separate subtle changes in the shape or placement of objects suggesting that irradiated rats over-completed patterns. In our fear conditioning task, loss of hippocampal neurogenesis resulted in an inability to associate a context with a fear-inducing event. This finding parallels those previously observed in the detrimental effect of chemotherapy, which is known to decrease neurogenesis (Mustafa et al., 2008), on contextual fear recall in male rats (Fardell et al., 2012). Together, these data suggest that irradiation-induced reduction of hippocampal neurogenesis does not impact all types of pattern separation processing equally. These data support the role of hippocampal neurogenesis in not only several types of pattern separation but also in the integration of contextual and aversive information.

The development of an object recognition task that involves the variation in the degree in similarity of objects (e.g., featural and geometric) or inter-trial delay (e.g., 3 hr and 1 min) is demonstrated here to be an excellent test of neurogenesis-dependent pattern separation ability. Similarly, object placement tasks that vary inter-trial delay (e.g., 3 hr and 1 min), can also be recommended or used conjointly with object recognition. These tasks are able to detect impairments affected by an irradiation-induced reduction in hippocampal neurogenesis and have the potential to detect improvements in pattern separation ability. Unlike previous tasks used to test pattern separation with fear conditioning, food motivation, or physical movement through a
maze, these tasks are able to measure levels of pattern separation ability without the likelihood of being confounded by non-cognitive differences in animals.


**Chapter 2:** Inherent and acquired aerobic fitness are determinants of enhanced hippocampal neurogenesis and pattern separation ability

The link between exercise and cognition has become well-accepted. In rodents, exercise has been shown to simulate all stages of hippocampal neurogenesis, from stem cell proliferation to neuronal survival and integration into the dentate gyrus. The production of these newborn neurons is thought to underlie exercise-induced improvement in learning and memory (van Praag, 2008) as well as stress regulation (Schoenfeld and Gould, 2013). However, exercise is a broad term that refers to physical activities aimed at improving overall wellness and health and can range from resistance training, to stretching, to aerobic activities. The majority of studies on the exercise-induced effects on the brain have used aerobic exercise in the form of daily physical activity (e.g., voluntary wheel running) or an exercise training regimen (e.g., endurance treadmill running). Intensity, duration, frequency, consistency, and time-of-day vary widely across exercise protocols, and recent work has shown that some of these factors can affect the magnitude of exercise’s benefits on neurogenesis and cognition (Leasure and Jones, 2008, Lou et al., 2008, Li et al., 2013). Aerobic exercises are thought to strengthen cardiorespiratory fitness (CRF) by increasing aerobic capacity, or the body’s ability to use oxygen (VO\(_2\) max) (Gaesser and Rich, 1984). However, aerobic capacity is rarely measured in studies of exercise effects on the brain and cognition, and therefore, the relationship between this component of exercise and neurocognition is largely unknown.

Cardiorespiratory fitness is a complex trait regulated by genetic and environmental factors. It has been suggested that CRF is influenced by two genetically-
controlled substrates: 1) an inherent factor that regulates baseline capacity for performing aerobic exercise and 2) an acquired, responsive element that allows aerobic capacity to change following a period of exercise training (Koch and Britton, 2001). In humans, there is significant variability in both the inherent cardiorespiratory fitness component as well as in the capacity to show increased cardiorespiratory fitness following a period of regimented exercise (Bouchard et al., 1998, Bouchard et al., 1999, Bouchard et al., 2000). One key research question is whether both inherent and acquired factors impact hippocampal neurogenesis and have functional consequences for neurogenesis-dependent cognition. Another important question is whether hippocampal neurogenesis is a specific target of altered aerobic capacity or whether differences in aerobic capacity has global effects throughout the brain, such as in other neurogenic niches like the subventricular zone (SVZ).

To address the influence of these two cardiovascular factors on adult neurogenesis and its functions, we have taken advantage of two groups of rats that have been selectively bred to model differences in inherent and acquired aerobic capacity. Both rat models were derived from a heterogeneous stock N:NIH founder population. The “inherent” model was produced by selective breeding for high or low intrinsic aerobic capacity (HCR and LCR, respectively) as assessed by maximal distance run on a treadmill fitness test (a measure of VO\textsubscript{2} max) (Koch and Britton, 2001). After 11 generations, HCR and LCR rats differed in their treadmill running capacity by 347\% in the absence of exercise training (Wisløff et al., 2005) and by 414\% by the 20th generation (Naples et al., 2010). The HCR/LCR rat model can be used to explore how large differences in aerobic capacity can influence brain and behavior. This model provides an additional advantage in that it isolates a cardiorespiratory fitness component that is heritable without being potentially confounded by exercise experience itself.
The “acquired” model involved selective breeding for the ability of rats to exhibit changes aerobic capacity after 8 weeks of incremental exercise training on a motorized treadmill (Koch et al., 2013). High response to training (HRT) rats greatly improve their running capacity following training while the low response to training (LRT) rats do not. Unlike the HCR/LCR model, HRT and LRT rats show similar aerobic capacities at a baseline; only the magnitude in change in aerobic capacity following treadmill training distinguishes the phenotypes. Also unlike HCR/LCR rats, HRT and LRT rats have a moderate degree of overlap in the response to training trait even after 15 generations of selection (Koch et al., 2013). The benefit of the HRT/LRT model is its ability to control exercise as a variable, with one group of animals highly responsive and the other highly resistant to the same training regimen. Positive effects of training on neurocognition in only HRT rats would support the necessity of increasing aerobic capacity, while positive effects of training in both HRT and LRT rats would imply that there are exercise-induced changes independent of aerobic capacity are beneficial to the brain.

To elucidate the influence of cardiorespiratory fitness on brain and behavior, HCR/LCR and HRT/LRT rat models of inherent and acquired fitness, respectively, were examined on measures of hippocampal neurogenesis and pattern separation ability, which are aspects of neurocognition previously shown to be enhanced by aerobic exercise. The potential for these variations in fitness to affect olfactory neurogenesis, neurotrophic factors, and hippocampal-independent behaviors, such as baseline activity and novel social interaction, was also explored.
Methods

**HCR and LCR rats**

Rats were selectively bred for low (LCR) or high (HCR) aerobic capacity for treadmill running from a heterogenous founder population for N:NIH stock rats (FND) at the Koch Britton Lab (University of Michigan, Ann Arbor, MI). The brains of 6- to 8-month-old female FND (n = 7) and 30th generation LCR (n = 4), and HCR (n = 4) rats were provided by the Thompson laboratory at Colorado State University (Fort Collins, CO). The body weights of the FND (214 ± 10.0 g) and LCR (215 ± 13.0 g) rats were similar and significantly greater ($F_{2,12} = 5.16$, $p < 0.05$) than that of HCR rats (177 ± 4.4 g). All rats were injected with 1-methyl-1-nitrosourea at 21 days of age to increase the likelihood of autochthonous mammary carcinomas. At sacrifice, the rats had tumor counts ranging from 0-4 and tumor biopsies were performed during necropsy; only rats with 0-2 benign tumors were used in this study. None of these rats were treadmill tested prior to sacrifice. The right hemisphere was post-fixed in 4% paraformaldehyde and the left hemisphere was flash frozen in liquid nitrogen, and the samples were mailed to Duke University.

A second cohort of LCR and HCR female rats from the 31st generation of selection were transported from the Koch Britton Lab (University of Michigan, Ann Arbor, MI) to the Duke University, where they were double-housed in ventilated shoebox cages in a climate controlled room with standard chow and water *ad libitum*. Prior to arrival, all rats were given a standard aerobic fitness test: HCR (1961 ± 52.35 m) and LCR (237 ± 19.44 m) rats differed in their treadmill running capacity by 826% ($p < 0.0001$). Rats were then behaviorally assessed for pattern separation ability on a parametric object recognition task, an object placement task, and context fear conditioning as previously described (Chapter 1). Two injections of BrdU (Sigma, St. Louis, MO)
separated by 8 hours were given to rats in order to label dividing cells. Half of each LCR and HCR rat groups were sacrificed 24 hours after the first BrdU injection for a cell proliferation timepoint; the remaining rats were sacrificed 1 month later for a cell survival timepoint. Rats were euthanized with CO₂ and brains were rapidly dissected. The right hemisphere of each rat was post-fixed in 4% paraformaldehyde for histological procedures, and the left hemisphere was flash frozen in isopentanol over dry ice and stored at −80°C. The right hemisphere was cryoprotected in 30% sucrose for 24 hrs prior to being sliced into 60 µm coronal sections on a freezing microtone through the rostral-caudal extent of the subventricular zone and the hippocampus or 60 µm sagittal sections through the olfactory bulb. Every fifth section was collected in 0.1% sodium azide to yield five series of sections. All animals underwent a 6 week quarantine and facility adaptation period prior to this study. All animal procedures were approved by the Institutional Animal Care and Use Committee of Duke University.

**HRT and LRT rats**

Rats selectively bred for low (LRT) or high (HRT) aerobic response to treadmill training were obtained from the Koch Britton Lab (University of Michigan, Ann Arbor, MI). Prior to arrival, rats were given a uniform 3 days/wk, 6-wk-long exercise training regimen and given a standard fitness test to measure maximal aerobic capacity for treadmill running as previously described (Koch et al., 2013): LRTs improved by only 24%, while HRTs improved on average by 77%. Female LRT (n = 8) and HRT (n = 9) rats from the 18th generation of selection were double-housed in ventilated shoebox cages in a climate controlled room with standard chow and water ad libitum at the Duke University vivarium. At 14 months-of-age, rats LRT and HRT rats were either handled or subjected to a uniform treadmill training protocol for 3 days/wk for 6 wks, that progressed moderately in speed (from 10-20 m/min) and duration (from 20-30 min).
Five injections of BrdU (Sigma, St. Louis, MO) were given every 8-12 hours at 150 mg/kg midway into the training regimen to label dividing cells. Fitness tests to measure aerobic capacity were given before, during, and after the treadmill training or handling period for all rats. Following the 6 weeks of training, rats were examined for anxiety-like behavior during a social interaction and for object recognition memory. Rats were behaviorally assessed for pattern separation ability on a parametric object recognition task as previously described (Chapter 1). Approximately one month following the final fitness test (and two months following BrdU injections), rats were euthanized with CO₂, and brains were rapidly dissected. The left hemisphere was post-fixed in 4% paraformaldehyde for histological procedures, and the right hemisphere was flash frozen in isopentanol over dry ice and stored at −80°. The left hemisphere was cryoprotected in 30% sucrose for 24 hrs prior to being sliced into 60 µm coronal sections on a freezing microtome through the rostral-caudal extent of the subventricular zone and the hippocampus. Every fifth section was collected in 0.1% sodium azide to yield five series of sections. All animals underwent a 6 week quarantine and facility adaptation period prior to this study. All animal procedures were approved by the Institutional Animal Care and Use Committee of Duke University.

**Immunohistochemistry**

Coronal sections (60 µm) containing the dorsal hippocampus or subventricular zone underwent immunohistochemistry for DCX (1:300 goat polyclonal, Santa Cruz Biotechnology, Dallas, Texas) and Ki67 (1:100 rabbit polyclonal, GeneTex, Irvine, CA) by first incubating in primary antibodies overnight at room temperature. Sections were then incubated in secondary antibody for 2 hours at room temperature using biotin anti-goat made in horse (1:200, Vector Laboratories, Burlingame, CA) or biotin anti-rabbit made in goat (1:200, Vector), respectively. Sections were incubated in Ready-to-Use
VECTASTAIN Elite ABC Reagent (Vector) and developed with SG Peroxidase Substrate (Vector) or DAB Peroxidase Substrate (Vector) for DCX or Ki67 immunoreactivity, respectively.

Sections containing the dorsal dentate gyrus (DG) of the hippocampus, subventricular zone (SVZ), or olfactory bulb (OB) were triple-labeled via fluorescent immunohistochemistry for BrdU, NeuN, and GFAP to characterize cells that had incorporated BrdU. Sections were denatured at 65° for 2 hours in Formamide/2x SSC and then at 37° in 2 N HCl for 30 min. Sections were incubated overnight at 4°C with sheep anti-BrdU (1:200, Abcam, Cambridge, England), mouse anti-NeuN (1:200, Millipore, Billerica, MA), and rabbit anti-GFAP (1:400, Dako, Glostrup, Denmark). Sections then were incubated for 2 hours at room temperature in Alexa Fluor 555 donkey anti-sheep (1:200, Life Technologies, Carlsbad, CA), Alexa Fluor 488 donkey anti-mouse (1:200, Life), and Alexa Fluor 647 donkey anti-rabbit (1:200, Life). Fluorescent images were captured using a Zeiss LSM 510 inverted confocal microscope in the Duke University Light Microscope Core Facility using an Argon/2 (488 nm), Diode (561 nm), and HeNe (633 nm) laser to detect Alexa Fluors 488, 555, and 647, respectively. For each hippocampal section, stacks of images (450 x 450 x 20 µm, centered approximately around the area of brightest immunofluorescence and using a 2 µm pinhole) were taken at 200x throughout the extent of the dentate gyrus. For each section of the OB, 2 image stacks (500 x 500 x 20 µm) of the granular cell layer were taken at 200x to sample the neurogenic area. For each SVZ section, one image (1000 µm x 1000 µm) was taken at 100x using a max pinhole to capture the dorsal extent of the lateral ventricle.

**Cell quantification**

Hippocampal sections containing Ki67+ cells that label proliferating cells and DCX+ cells that label young neurons were quantified using Stereo Investigator (MBF
Bioscience, Williston, VT). Contours were drawn around the region that encompassed the dorsal and ventral blades of the DG, including the granule cell layer and subgranular zone using a 40x objective lens on a Nikon light microscope. Volume estimates were generated using the optical fractionator and according to Cavalleri’s principle (Mouton, 2002). There were no significant differences in DG volume obtained from these contours across experimental conditions. We used a modified fractionator principle to move exhaustively throughout each region, using an optical dissector height of 20 µm with a 2 µm guard zone to avoid over-sampling between brain sections. To represent the extent of the dorsal DG, 5 sections (each separated by a step of 300 µm) per rat were quantified. Data are represented as the number of cells per dorsal DG in one hemisphere.

The number of Ki67+ cells in the anterior part of the SVZ was quantified similarly to that in the dorsal dentate gyrus, but with 3 sections per rat. Contours were drawn around the extent of immunoreactivity surrounding the lateral ventricle using a 40x objective lens on a Nikon light microscope. Volume estimates revealed a larger SVZ in HCR compared to LCR rats ($p < 0.05$) likely due to contours being based off of the area of ki67+ cells in the region rather than the size of the lateral ventricle itself. Data are represented as the number of cells per anterior SVZ in one hemisphere.

Confocal images of the DG (5 sections per rat, with 4-7 Z-stacks each) or the subgranular zone of the OB (3 sections per rat, with 2 Z-stacks each) were analyzed using the Cell Counter plugin on ImageJ (NIH, Bethesda, MD). BrdU+ cells (red) were counted and categorized as co-expressing NeuN (green), GFAP (blue), or other (indeterminable). Counts for the DG were multiplied by a factor of 12.5 to create an DG estimate comparable to the calculation used by Stereo Investigator from the optical fractionator principle. Data for the OB counts are represented as the average counts per Z-stack to represent a sample region-of-interest (ROI) for each rat OB. For each fluorescent SVZ
image, two circular ROIs (125 µm diameter) were drawn over two adjacent areas of the lateral ventricle. The number of BrdU+ cells were counted exhaustively in each ROI and data area represented as the average number of BrdU+ cells per SVZ ROI.

RT-PCR

From brains of the FND, LCR, and HCR rats were provided by the Thompson laboratory, 3 frozen tissue punches were taken from the dorsal hippocampal region of the left-hemisphere. The tissue samples were then homogenized following manufacturer’s instructions with RNA extraction buffer (TRIZOL reagent, Life Technologies) to yield total RNA. The total RNA was reverse transcribed with poly-dT oligonucleotides and SuperScipt II (Life Technologies, Inc.) following the manufacturer’s instructions. To start, 5 µl of RNA were treated with DNase, and this mixture was combined with 2.5 µl of oligo(deoxythymidine) primer, and 500 µl of each dNTP. Each tube was then heated at 65°C for 5 minutes. This was followed by the addition of 1x RT buffer, 0.01 M dithiothreitol, 5 mM MgCl₂, and 2 U/µl RNaseOUT ribonuclease inhibitor. The resulting mixture was then heated again to 42°C for 5 minutes. 50 µl of Superscript II reverse transcriptase was added before the final heating to 42°C for 50 minutes, followed by 70°C for 15 minutes. All of heating in the preceding steps were performed in an Eppendorf Realplex Mastercycler machine. Gene transcripts were then quantified using a real-time PCR quantification system (Eppendorf Realplex Mastercycler) and then analyzed on Excel for fold change of target mRNA to the housekeeping, gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences for GAPDH (Okamoto et al., 2012), glucocorticoid receptor (GR) (Nishimura et al., 2002) and mineralocorticoid receptor (MR) (Sasaki et al., 2013), brain derived neurotrophic factor (BDNF) (Mitsukawa et al., 2006), vascular endothelial growth factor
A (VEGF-A) (Aoi et al., 2008), insulin-like growth factor 1 (IGF-1) (Wong-Goodrich et al., 2008), androgen receptor (AR) (Okamoto et al., 2012), and estrogen receptor alpha (ESR-1) (Okamoto et al., 2012) were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table 2.1. Each reaction reagent was prepared as a master mix, and then aliquot into three individual wells. The cDNA was first denatured at 95°C for 5 minutes, followed by amplification preformed over 40 cycles of denaturation (95°C for 15 s), annealing (60°C for 45 s) and elongation (72°C for 60 s). The amplification step was monitored via the fluorimetric intensity of SYBR Green I at the end of each elongation phase, and the specificity of the amplification was monitored via analysis of the melting curve. Negative control experiments were used in order to verify the signal was derived from the cDNA.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GTG CCA AAA GGG TCA TCA TCT C</td>
<td>GGT TCA CAC CCA TCA CAA ACA TG</td>
</tr>
<tr>
<td>GR</td>
<td>TTC GAA GGA AAA ACT GCC CAG</td>
<td>CGA GCT TCA AGG TTC ATT CCA</td>
</tr>
<tr>
<td>MR</td>
<td>GGC AGC TGC AAA GTC TTC TT</td>
<td>GAC AGT TCT TTC GCC GAA TC</td>
</tr>
<tr>
<td>BDNF</td>
<td>CCA TAA GGA CGC GGA CTT GT</td>
<td>GAG GCT CCA AAG GCA CTT GA</td>
</tr>
<tr>
<td>VEGF</td>
<td>CAC TGG ACC CTG GCT TTA CTG</td>
<td>CAC TCC AGG GCT TCA TCA TTG</td>
</tr>
<tr>
<td>IGF-1</td>
<td>GTG GAC GCT CTT CAG TTC GT</td>
<td>GCT TCC TT TCT TGT GTG TCG ATA G</td>
</tr>
<tr>
<td>AR</td>
<td>AGT ACC AGG GAC CAC GTT TTA C</td>
<td>CAC AGA TCA GGC AGG TCT TCT</td>
</tr>
<tr>
<td>ESR-1</td>
<td>GCC CGC AGC TCA AGA TG</td>
<td>CTT GCT GTT TGC CAC GTA CAC</td>
</tr>
</tbody>
</table>

**Statistical analysis**

All data are presented as means ± standard error. Using an α level of 0.05, data were analyzed using Student’s t-test, paired t-test, ANOVA, RMANOVA, and *a priori* comparisons where appropriate with JMP Pro v10.0.2 (SAS Institute, Cary, NC). NS indicates a *p* value that was not statistically significant.
Results

**Rats with inherent high aerobic capacity have enhanced hippocampal neuronal survival**

By the 31st generation of selection for high and low aerobic capacity, HCR rats exhibited a maximal aerobic capacity of $1961 \pm 52$ m, which was eight-fold higher than LCR rats that ran $237 \pm 19$ m on a test of maximal aerobic capacity at 6 months-of-age (Fig. 2.1A). Remarkably, HCR rats exhibited more than double (227%) the number of DCX+ young neurons in the dentate gyrus of the hippocampus compared to LCR rats ($p < 0.001$) at the time of sacrifice at 11-13 months-of-age (Fig. 2.1B & 2.1C). The number of DCX+ cells was significantly correlated with maximal aerobic capacity (Pearson correlation, $p < 0.001$). In contrast, HCR and LCR rats showed no significant differences in Ki67+ proliferating cells at the time of sacrifice, suggesting that the effect of inherent aerobic capacity on neurogenesis is specific to neuronal survival not cell proliferation (Fig. 2.1D).
Figure 2.1: Rats selectively bred for high aerobic capacity have a higher rate of neuronal survival in the hippocampus compared to low aerobic capacity rats. A) Aerobic capacity (maximal running distance, m) on a fitness test for LCR and HCR rats, *p < 0.0001. B) Number of DCX+ young neurons in the DG of LCR and HCR rats, *p < 0.001. C) Photomicrographs of DCX+ neurons (indicated by arrows) in the DG of a representative LCR and HCR rat. D) Number of Ki67+ proliferating cells in the DG of a LCR and HCR rat. E) Counts showing 24 hr proliferation or 1 month survival of BrdU+ cells in the DG of LCR and HCR rats. *1 month survival significantly different from 24 hr proliferation, p < 0.05. F) Proportion of BrdU+ cells in the DG that coexpress neuronal (NeuN) or astroglial (GFAP) markers following 1 month survival. *NeuN+ in HCR rats significantly different than LCR rats, p < 0.05.
Characterization of BrdU+ cells in the DG at the 24 hour and 1 month post-BrdU injection timepoints was conducted to confirm a lack of differences in the rate of proliferation and to determine the fate of DG cells born 1 month prior to sacrifice (see example, Fig. 2.2). Consistent with the Ki67 proliferation findings, 1 day following BrdU administration, there were no significant differences between the number of BrdU+ cells in the DG of LCR and HCR rats (Fig. 2.1E). However, 1 month after BrdU administration, LCR rats had significantly fewer BrdU+ cells ($p < 0.05$), while HCR rats did not show a significant reduction in the number of BrdU+ labeled cells, suggesting that the HCR phenotype enhanced the survival of new cells (Fig. 2.1E). While there was no significant difference in the total number of BrdU+ cells between HCR and LCR rats at the 1 month timepoint, the number of these BrdU+ cells that expressed an NeuN+ neuronal phenotype was greater (by 425%) in HCR rats compared to LCR rats ($p < 0.05$) (Fig. 2.1F). There were no differences in the proportion of BrdU+ cells that expressed a GFAP+ astroglial or other phenotype. Taken together, these results suggest that inherent aerobic capacity has a large influence on survival and maturation of newborn neurons in the dentate gyrus, but not baseline cell proliferation.
Figure 2.2: Triple-label fluorescent immunohistochemistry for NeuN, GFAP, and BrdU. Confocal images from the dentate gyrus of an LCR rat. Top left, NeuN+ adult neurons (green). Top right, GFAP+ astroglia (blue). Bottom left, BrdU+ cells (red). Bottom right, overlay image indicating one BrdU+/GFAP+ cell (top arrow) and one BrdU+/NeuN+ cell (bottom arrow). Scale bar indicates 50 µm.
Selective breeding for high aerobic capacity also bred for an increase in neurogenesis divergent from the founder population

To examine how hippocampal neurogenesis was altered by selective breeding for aerobic capacity, the brains of 6- to 8-months-old HCR and LCR rats from the 30th generation were examined alongside aged-matched rats from the heterogeneous N:NIH stock founder population (FND). HCR rats had three-fold more DCX+ young neurons in the DG compared to LCR or founder rats ($F_{2,8} = 16.00, p < 0.01$) (Fig. 2.3A & C). Also consistent with the initial finding, there were no significant differences in the number of proliferating cells immunopositive for Ki67 ($F_{2,9} = 0.67, \text{NS}$) (Fig. 2.3B & C). Together, these results suggest that selecting for high running capacity also selects for a factor that is important for increased survival, but not proliferation, of new neurons in the hippocampus.
Figure 2.3: Selection for high aerobic capacity conferred a phenotype of high levels of neurogenesis compared to the heterogeneous founder population. 

A) Number of DCX+ young neurons in the dentate gyrus (DG) of 6- to 8-month-old rats from the founder population (FND) and the 30th generation of selection for intrinsic low (LCR) or high (HCR) capacity for running. *p < 0.0001. 

B) Number of Ki67+ proliferating cells in the DG of FND, LCR, and HCR rats. 

C) Photomicrographs of representative DCX (top) and Ki67 (bottom) stains from a FND, LCR, and HCR rat.
Selective breeding for high and low aerobic capacity has little effect on hippocampal glucocorticoid, neurotrophic, and hormonal factors

Despite the large differences in neurogenesis detected among HCR, LCR, and FND rats, we found very few differences in a number of factors that have previously implicated as important regulators of neurogenesis. For example, there were no significant differences in the fold change of in the hippocampal mRNA for glucocorticoid receptor (GR) or mineralcorticoid receptor (MR) relative to the housekeeping gene GAPDH found among FND, LCR, and HCR rats (Fig. 2.4A). However, HCR rats exhibited a significantly greater GR:MR ratio of mRNA expression ($F_{2,11} = 9.82, p < 0.01$) (Fig. 2.4B). Surprisingly, there were also no significant differences in the hippocampal mRNA expression of brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), or vascular endothelial growth factor A (VEGF-A) (Fig. 2.4C) nor in the mRNA expression of androgen receptor (AR) or estrogen receptor alpha (ESR-1) (Fig. 2.4D).
Figure 2.4: Gene expression for potential neurogenic factors in FND, LCR, and HCR rats. A) MR and GR mRNA fold change relative to GAPDH. B) Ratio of GR to MR fold change. *HCR rats different from FND and LCR rats, p < 0.01. C) BDNF, IGF-1, and VEGF-A mRNA fold change relative to GAPDH. D) AR and ESR-1 mRNA fold change relative to GAPDH.
**Inherent high aerobic capacity results in increased SVZ proliferation but not OB neuronal survival**

To determine whether inherent cardiorespiratory fitness has a specific influence on the hippocampus or more global effects, we examined neurogenesis in the subventricular zone-olfactory bulb (SVZ-OB) pathway. One day following BrdU administration, HCR rats had significantly more BrdU+ cells in the SVZ compared to LCR rats \((F_{3,12} = 98.37, \text{main effect of strain}, p < 0.01)\) (Fig. 2.5A & E). Consistent with this finding of increased SVZ proliferation, we also found that HCR rats had significantly more Ki67+ proliferating cells than LCR rats at the time of sacrifice \((p < 0.01)\) (Fig. 2.5B). By 1 month following BrdU administration, there were very few BrdU+ cells remaining in the SVZ for both LCR and HCR rats \((F_{3,12} = 98.37, \text{main effect of timepoint}, p < 0.0001)\) (Fig. 2.5C & E). By comparison, the granular cell layer (GCL) of the olfactory bulb, which did not show a large amount of 24 hr proliferation, showed a dramatic increase in the number of BrdU+ cells at the 1 month timepoint \((F_{3,12} = 3.37, \text{main effect of timepoint}, p < 0.01)\) due to the migration of cells from the SVZ through the rostral migratory stream (RMS) to the olfactory bulb (OB) (Fig. 2.5C & E). The proportion of BrdU+ cells in the olfactory bulb that survived and matured into NeuN+ neurons was not significantly different between HCR and LCR rats (Fig. 2.5D). While differences in inherent aerobic capacity may alter the proliferation of cells in the SVZ, effects of inherent aerobic capacity on neurogenesis are not evident in the olfactory bulb and thus, appear to be specific to the hippocampus.
Figure 2.5: Characterization of neurogenesis in the SVZ-OB pathway in rats selected for high and low aerobic capacity. A) BrdU counts 24 hr or 1 month following injections for a sample region-of-interest (ROI) in the SVZ of LCR and HCR rats. *24 hr proliferation is greater in HCRs than LCRs, p < 0.01. †Fewer cells at the 1 mo timepoint, p < 0.0001. B) Confirmation of number of proliferating cells using Ki67 in the SVZ of LCR and HCR rats. *p < 0.01. C) Number of BrdU+ cells 24 hrs or 1 mo post-injections in an ROI in the granular cell layer of the OB of LCR and HCR rats. *More cells in the OB at 1 mo, p < 0.01. D) Proportion of BrdU+ cells in the OB that coexpress neuronal (NeuN) or astroglial (GFAP) markers following 1 mo survival. E) Images of triple-label immunofluorescence for BrdU+ newborn cells (red), GFAP+ astroglia (blue), and NeuN+ adult neurons (green) in the SVZ and olfactory bulb (OB) at the 24 hr and 1 mo timepoints from representative LCR and HCR rats. Scale bars indicate 100 µm and 50 µm for the SVZ and OB images, respectively.
Inherent high aerobic capacity confers a phenotype of high accuracy on pattern separation tasks

The determine if differences in inherent levels of neurogenesis between HCR and LCR rats modified their cognitive ability, rats were trained on a variation of a standard object recognition task that we have shown to be sensitive to alterations in hippocampal neurogenesis (see Chapter 1). In a challenging pattern separation task, where only a small feature of an object was modified between training and test trials after a 1 min delay, HCR rats, but not LCR rats, spent significantly more time investigating the novel object compared to the familiar object ($p < 0.05$ by paired two-tailed t-test) (Fig. 2.6A). When the object change between training and testing was a more obvious geometric inversion of the original object, HCRs ($p < 0.02$) again displayed accurate pattern separation ability (Fig. 2.6B). While the performance of LCR rats on this geometric discrimination did not reach significance by a two-tailed t-test, the assumption that rats spend more time in the direction of the novel object over the familiar allowed for the application of a one-tailed analysis, which revealed that LCR rats spent significantly more time with an object that underwent a geometric inversion ($p < 0.05$).

To evaluate whether differences in pattern separation accuracy might be due to differences in experience with the familiar objects, the time spent with the two identical objects used in the training trials was also quantified. There were no significant differences between LCR and HCR rats in time spent with the left object, the right object, or both objects total during the training trials. However, there was significantly greater variability ($p < 0.001$ by $F$-test) within the LCRs (48.72 ± 8.84 s) compared to HCRs (35.88 ± 1.70 s) in the total average time spent investigating the objects during the training, which appeared to be in the direction of more LCR rats with high exploratory behavior. Therefore, the relatively higher accuracy of the HCR rats on the test trials...
cannot be attributed to HCR rats spending more time with the trained objects compared to LCR rats.

Paralleling the results from the object recognition tasks, in a challenging version of an object placement task, only the HCRs ($p < 0.05$) spent significantly more time investigating the moved object when the delay between training and testing was 3 hrs ($p < 0.05$) (Fig. 2.6C). However, when the delay was shortened to just 1 min, both HCRs and LCRs ($p < 0.05$) spent significantly more time investigating the moved object (Fig. 2.6D). Taken together, these data reveal that HCR rats are able to code small featural differences between objects more accurately and retain small changes in object positions for a longer duration than LCR rats.
Figure 2.6: Rats selectively bred for high aerobic capacity show enhanced performance on neurogenesis-dependent pattern separation tasks. Time spent (s) with familiar and novel objects during the 1st minute of the 3-minute test following a 1 minute delay for a featural (A) or geometric (B) configuration. *p < 0.05 by two-tailed paired t-test, ^p < 0.05 by one-tailed paired t-test. Time spent (s) with familiar and novel objects during the 1st minute of the 3-minute test following a 3 hour (C) or 1 minute (D) delay for a change in object placement. *p < 0.05.
Inherent high aerobic capacity rats display stronger context fear associations

Because contextual fear conditioning has been shown to be influenced by hippocampal neurogenesis (see Chapter 1), contextual fear conditioning was also evaluated in these rats 48 hrs following a one-trial experience in a novel chamber where a tone was followed by an aversive foot shock (Fig. 2.7A). LCR and HCR rats displayed similarly high levels of freezing to the original context (a hippocampal-dependent task) (Fig. 2.7 B). When placed in a cage with a slightly altered floor, shape, odor, and lighting compared to the original fear conditioning context, LCR rats showed minimal levels of freezing, while HCR rats displayed high levels of freezing that was no different than in the original context and significantly greater than that in LCR rats ($p < 0.01$). HCR also rats froze significantly more ($p < 0.05$) than LCR rats to the conditioned tone, which is known to be an amygdala-dependent task (Goosens and Maren, 2001). Overall, HCR rats were more responsive to cues that could be associated with an aversive event.
Figure 2.7: HCR, but not LCR, rats generalized conditioned fear and made stronger US-CS associations. A) Fear-conditioning protocol in which freezing behavior was scored during a 6 minute test in the conditioned context (hippocampus dependent), a 3 minute test in a completely different context (generalization), and a 3 minute tone test in the different context (amygdala-dependent) following a 48-hour delay from the training experience. B) Percent freezing to the original context, different context, and tone. *p < 0.01, ^p < 0.05.

Selective breeding for high response to training affects neurogenesis and pattern separation ability

To determine whether improved aerobic capacity might be necessary for exercise-induced improvements in hippocampal function, we used an animal model in which rats were selectively bred for high (HRT) versus low (LRT) aerobic response to treadmill training (Koch et al., 2013). For 3 days/wk for 6 wks, 14-month-old female LRT and HRT
rats from the 16th selected generation were either handled or subjected to an endurance training protocol that progressed moderately in speed (from 10-20 m/min) and duration (from 20-30 min) and was given identically to all trained rats. Trained LRT rats (LRT-T) ran slightly (32 ± 13.0%) farther on the final compared to initial fitness test ($p < 0.05$), while trained HRT rats (HRT-T) showed a drastic (91 ± 24.1%) increase in aerobic capacity ($p < 0.01$), as expected for their phenotype (Fig. 2.8A).

One month following the cessation of exercise training, HRT-T, but not LRT-T, rats showed a significant increase (by 34%) in the number of DCX+ young neurons in the DG compared to their sedentary counterparts ($p < 0.05$) (Fig. 2.8B & C). Neither training nor selective breeding influenced the number of Ki67+ proliferating cells in the DG (Fig. 2.8D). BrdU injections were administered midway in the training protocol to track the survival of newborn cells. While there were no significant differences in the total number of BrdU+ cells in the DG across groups (Fig. 2.8E) or in the proportion that survived into neurons (Fig. 2.8F), a pattern of increased neurogenesis specific to the HRT-T rats is consistent across all measures of neurogenesis in the HRT/LRT model.
Figure 2.8: Only rats that show a high aerobic response to training show an increase in hippocampal neurogenesis. 

A) Maximal aerobic capacity (m) at the initial, midpoint, and final fit tests throughout a 6-week-long standardized exercise regimen. Trained LRT rats (LRT-T, *p < 0.05) and trained HRT rats (HRT-T, *p < 0.01) ran farther on the final compared to initial fit test. 

B) HRT-T, but not LRT-T, rats showed increased DCX+ young neurons in the DG compared to their sedentary counterparts (*p < 0.05). 

C) Photomicrographs of DCX+ neurons (indicated by arrows) in the DG of a representative LRT, LRT-T, HRT, and HRT-T rat. No significant differences were found in the number of Ki67+ proliferating cells (D), 1 mo survival of BrdU+ cells (E), or in the proportion of BrdU+ cells that coexpress NeuN or GFAP (D).
**A high response to training is needed for improved pattern separation accuracy**

Trained and untrained HRT and LRT rats were evaluated on the pattern separation ability. Only HRT-T rats spent significantly more time investigating an object that had been slightly modified by a featural change compared to a familiar object following a 1 min delay ($p < 0.001$), indicating a highly accurate pattern separation ability (Fig. 2.9A). While untrained HRT rats did not demonstrate significant recognition on this difficult task by a two-tailed paired t-test, the prediction that these rats should favor novelty allowed for the use of a one-tailed paired t-test, which demonstrated that untrained HRT rats recognized the featural change on average ($p < 0.05$). Performance on this task (time spent with novel – familiar) was positively correlated with the number of DCX+ young neurons in the hippocampus (Pearson correlation, $p < 0.01$) as well as change in maximal aerobic capacity ($p < 0.05$). Both untrained HRT ($p < 0.05$) and HRT-T ($p < 0.05$) rats demonstrated accurate performance on a less difficult pattern separation task where an object underwent a geometric inversion (Fig. 2.9). LRT rats, regardless of training, did not significantly discriminate between the objects on either pattern separation task.
Inherent and acquired aerobic fitness have no major effects on a standard object recognition task

In order to determine whether hippocampal-independent cognition is affected by differences in aerobic capacity, rats from the HCR/LCR and HRT/LRT models were also evaluated on a task to test standard object recognition, which is thought to be hippocampal-independent due to the dissimilarities between the objects used (Fig. 2.10A). HCR ($p < 0.05$) and LCR ($p < 0.05$) rats similarly showed accurate recognition of the novel object over the familiar (Fig. 2.10B). Performance of LRT ($p < 0.05$), LRT-trained ($p < 0.05$), HRT ($p < 0.05$), and HRT-trained rats ($p < 0.01$), was also similar and highly accurate.
Figure 2.10: Standard object recognition for rats bred for inherent and acquired differences in aerobic capacity. A) Diagram for an example standard object recognition task with a soda can (familiar object) and a coffee mug (novel object). Average time spent with familiar verses novel object was timed throughout the 3 minute object recognition test following a 1 minute delay from training with two identical objects. B) Performance of LCR and HCR rats. *p < 0.05. C) Performance of LRT, LRT-trained, HRT, and HRT-trained rats. **p < 0.01. *p < 0.05.

Inherent and acquired alterations in aerobic capacity affect baseline locomotor activity

Rats from the HCR/LCR and HRT/LRT models were placed in an activity box for 1 trial per day to evaluate locomotion and habituation to novel environment over the course of 3 days. Overall, across all minute intervals from day 1 to 3, LCR rats traveled a greater distance (cm) compared to HCR rats ($F_{1,14} = 10.11, p < 0.0067$); this differences was similarly evident within day 1 ($F_{1,14} = 5.80, p < 0.05$) and day 2 ($F_{1,14} = 7.34, p < 0.05$) (Fig. 2.11A). Locomotor activity appeared to be more similar across untrained and trained HRT and LRT groups. Although, there was a significant strain x training interaction on the final minute for day 3 (between subjects $F_{3,13} = 1.35$, NS; within subjects from min 2-3, $F_{1,13} = 4.92, p < 0.05$) (Fig. 2.11B).
Figure 2.11: Activity box data for rats bred for inherent and acquired differences in aerobic capacity. 

A) Average distance traveled (cm) for LCR and HCR rats across the span of 3 days in a novel activity box. *Main effect of strain between subjects across time, $p < 0.05$. 

B) Average distance traveled (cm) for LRT, LRT-trained, HRT, and HRT-trained rats across the span of 3 days in a novel activity box. *Significant strain x training interaction effect. $p < 0.05$. 
Inherent and acquired alterations in aerobic capacity influence social interaction behavior

In order to determine whether selectively-bred differences in inherent or acquired aerobic capacity might affect social interaction, a measure of anxiety-like behavior, rats were habituated to an arena and given a social interaction trial with a novel conspecific that could only be investigated through a perforated cage (Fig. 2.12A). Amount time tracked in the social interaction zone and avoidance corners were recorded for the HCR/LCR and HRT/LRT rat models of aerobic fitness. There were no significant differences between HCR/LCR rats in time spent in the avoidance corners, although LCR rats showed greater social interaction than HCR rats at during the second minute (p < 0.05). While all HRT/LRT groups showed a similar high level of investigation in the first minute of the trial, HRT rats seemed to show overall greater habituation and there was a significant strain x training interaction effect though the first minute interval (between subjects $F_{3,13} = 3.35$, NS; within subjects, time*strain, $F_{1,13} = 6.74$, p < 0.05, time*strain*train, from min 1-2, $F_{1,13} = 5.46$, p < 0.05). There were no significant effects of HRT/LRT strain or training on time spent in the avoidance corners.
Figure 2.12: Novel social interaction in rats bred for inherent and acquired differences in aerobic capacity. A) Social interaction test diagram. Each rat was habituated to the arena for 3 minutes. Following a 1 min delay, the rat (black) was placed back into the arena with a novel conspecific (light gray rat) in a perforated cage for 3 min. Interaction zone and avoidance corners are highlighted in light gray. B) Average time (s) LCR and HCR rats spent in interaction zone (left) and avoidance corners (right). *LCR rats greater than HCR rats at during the second minute, $p < 0.05$. C) Average time (s) LRT, LRT-trained, HRT, and HRT-trained rats spent in interaction zone (left) and avoidance corners (right). *Significant main effect of strain, $p < 0.05$. **Significant strain x training interaction effect from min 1 to 2, $p < 0.05$. 
Discussion

The present study demonstrates that both inherent and acquired cardiorespiratory fitness modulate hippocampal neurogenesis and cognition that is thought to be influenced by numbers of new neurons in the hippocampus. While many prior studies have shown that voluntary wheel running and treadmill training increase hippocampal neurogenesis and cognitive function, the current study points to aerobic capacity as a key component of exercise that appears to dramatically influence neurocognition. Exercise without changes in aerobic capacity does not influence neurogenesis or cognition and high aerobic capacity, even without exercise, improves neurocognitive function.

There is a strong correlation between aerobic capacity and adult neurogenesis, which has a direct effect on cognition. Parallel to their differences in inherent aerobic capacity, rats selectively bred for high aerobic capacity have 2- to 3-fold more DCX+ young neurons (and a 4- to 5-fold higher rate of NeuN+ neuronal maturation of newborn cells) in the adult hippocampus compared to rats bred for low aerobic capacity, a trait that appears to have diverged from the heterogeneous founder population. High capacity runners also demonstrate high accuracy on pattern separation tasks, a cognitive ability known to be dependent on levels of neurogenesis. These findings reveal that high aerobic capacity, even in the absence of exercise, leads to high levels of hippocampal plasticity and improved cognitive function. Moreover, this boost in neurogenesis is arguable more robust than any previously reported effect of exercise itself.

While there is greater overlap in rats selectively bred for the acquired component of CRF, only rats that display a high aerobic response to exercise training showed an improvement in hippocampal neurogenesis and accuracy on pattern separation tasks. Low aerobic responders who underwent the same exercise regimen did not significantly
increase levels of neurogenesis or pattern separation ability. These data strongly suggest that an exercise-induced increase in aerobic capacity, rather than exercise, *per se*, is necessary for enhancing hippocampal neurocognition. Taken together, these data provide strong evidence that inherent and acquired aerobic capacity, which are crucial components of cardiorespiratory fitness, are also key factors that modulate hippocampal neurogenesis and cognition.

Variations in aerobic capacity appear to impact only the survival of young neurons in the hippocampus. In contrast, wheel running and treadmill training of several rat and mouse strains appears to alter both cell proliferation and neuronal survival (van Praag et al., 1999, van Praag, 2008). Although, some reports of the effects of exercise suggest that neuronal survival is the key target, particularly in terms of neuroprotection where a loss of neural stem cells might be compensated by mechanisms to enhance neuronal survival of the remaining cells that do proliferate (Wong-Goodrich et al., 2010). It is also possible that exercise-induced effects on proliferation can be attributed to an increase in vascular cells (angiogenesis) and glia (gliogenesis), rather than increasing the genesis of neuroblasts that will become neurons. The density of microvessels in the dentate gyrus, for instance, positively correlates with the number of DCX+ young neurons but not the number of Ki67+ proliferating cells following 10 days of voluntary wheel running in mice (Van der Borght et al., 2009). The gliogenesis of GFAP+ astrocytes in the hippocampus, which occurs in parallel to adult neurogenesis, also seems to be regulated somewhat independently of neurogenesis (Steiner et al., 2004). Voluntary running can induce the genesis of two populations of GFAP+ cells: one that is S-100β-negative, a putative indicator of astrocyte-like neural progenitor cells, and one that is S-100β-positive. By comparison, very few newborn microglia and no new oligodendrocytes are detected in the hippocampus. However, aerobic exercise has been
shown to increase the genesis of astrocytes and oligodendrocytes in the mouse prefrontal cortex after wheel running (Mandyam et al., 2007) and the genesis of astrocytes in the cortex and striatum of rats where extensive angiogenesis has been found after treadmill running (Li et al., 2005). Rather than the quantity, alterations in the cytokine expression profile of microglia have also been shown to regulate the activation of hippocampal neural precursor proliferation in response to exercise (Vukovic et al., 2012). While there could be small differences in the rate of proliferation in non-neuronal cells or the activation of microglia, as well as changes in these cell types following exercise, the inherent differences between HCR and LCR rats point to a particular role for aerobic capacity in the neuronal survival stage of neurogenesis and not total cell proliferation in the hippocampus.

Data from our HRT/LRT model of acquired CRF also support the role for aerobic capacity in neuronal survival rather than cell proliferation. Although, it is possible that we did not capture the right timepoints for detecting training effects on cell proliferation. Training may have increased cell division, but its effects on proliferation were not evident one month following the cessation of treadmill exercise training when the rats were sacrificed. In contrast, the number of DCX+ young neurons in HRT rats that were highly responsive to training was still elevated one month period following training, pointing to a strong and perhaps lasting effect of training on neuronal survival. Cognitive behavior was also assessed during this post-exercise period and only high responders exhibited an improved pattern separation ability that was positively correlated with the number of young neurons. Therefore, exercise may induce a number of factors that stimulate proliferation and survival, but an acquired aerobic capacity component produces enduring levels of neuronal survival that are responsible for improving neurogenesis-dependent cognition.
The anterior SVZ is a specialized region that generates neuroblasts throughout adulthood that migrate through the rostral migratory stream (RMS) into the olfactory bulb (OB) (Suzuki and Goldman, 2003). Our finding suggest that in the SVZ, unlike the SGZ of the hippocampus, responds to high aerobic capacity with increased cell proliferation. This finding of more SVZ proliferation in HCR than LCR rats is consistent with some previous reports of exercise-induced SVZ proliferation (Yasuhara et al., 2007, Tajiri et al., 2010), although the effect of exercise on SVZ proliferation is not as reliable as its effect on hippocampal neurogenesis (Brown et al., 2003). While high capacity runners show increased SVZ proliferation compared to low capacity runners, this enhancement does not seem to translate to increased neuronal survival in the olfactory bulb. This finding is also consistent with reports that exercise does not alter olfactory bulb neurogenesis (Brown et al., 2003). Although, it might also be possible that while SVZ proliferation is increased by changes in aerobic capacity, without sufficient olfactory stimulation, few of these cells are able to establish connections in the bulb, as this is known to be an activity-dependent process (Cecchi et al., 2001). It is possible that exercise-induced new neurons from the SVZ migrate to other brain regions and establish functional connections. For example, there is evidence that SVZ proliferation produces new neurons that migrate to other regions of the rodent brain, such as the hypothalamus (Kokoeva et al., 2005), amygdala (Fowler et al., 2008), and the striatum (Pencea et al., 2001), which was most recently demonstrated to also occur in humans (Ernst et al., 2014). While the significance and functional relevancy of neurogenesis in these regions is still unknown, it is certainly possible that exercise-induced cells may be added to these regions.

A few cognitive behaviors had been previously evaluated in the HCR/LCR rats model. For instance, HCR rats were partially protected from a decline in freezing
behavior to the context in a trace-fear condition task 3 days following aseptic trauma involving tibial fracture (Su et al., 2012). This finding that HCR rats are resilient to decreasing freezing behavior is similar to our current observation of HCR displaying high levels of freezing to a context that was dissimilar from the original context in which a foot shock was delivered compared to LCR rats in contextual fear conditioning. We also showed that HCR rats display higher levels of freezing to the cued tone compared to LCR rats. To further support this behavioral difference, HCRs, compared to LCRs, stay longer in a bright chamber and avoid a dark chamber where they were previously shocked on a passive-avoidance task (Sarga et al., 2013). These results were initially surprising considering that HCR rats have more neurogenesis in LCR rats, and enhancements in neurogenesis have previously been shown to decrease generalization of conditioned fear to a similar context across multiple trials (Sahay et al., 2011a). However, while one interpretation of our finding that HCR rats show more generalization of conditioned fear following a single trial, an alternative interpretation is that this behavior is demonstrative of stronger memory traces and improved spatial memory in HCR rats. This alternative interpretation is also consistent with our previous finding that rats with low neurogenesis following whole-brain irradiation do not generalize fear or show much freezing even to the original context (Chapter 1). It seems possible that high levels of neurogenesis result in stronger fear-context associations following a one-trial experience, while low levels reduce the formation of associations. A repeated-exposure contextual fear conditioning paradigm like what was performed in Sahay et al. (2011a) might reveal that HCR rats generalize less, rather than more, compared to LCR rats when given multiple opportunities to distinguish between an original context where foot shocks are given and a similar context where foot shocks are never given. It is plausible that higher numbers of easily-excitable neurons, like in HCR rats, create an enriched
memory trace that can both 1) be compared to a similar and therefore, potentially dangerous, other context, in which generalizing conditioned fear is advantageous and 2) facilitate discrimination between a similar contexts, allowing for quick pattern separation and extinction of the fear response to that different context across multiple trials. In other words, it is entirely possible and perhaps likely that HCR rats recognized the differences in the original and different contexts used in the current study, but were advantaged to respond to its similarities. The accurate pattern separation abilities of HCR rats observed on object recognition and object placements tasks used in this study demonstrate that HCR rats are able to detect small differences and similarities (novelty and familiarity) in a spatial environment when it is not confounded by fear. It is possible that the responsivity of high aerobic capacity rats to stressful stimuli, including novelty, allows a cognitive advantage.

Along the same lines, HCR rats seem to be more responsive to stressful stimuli compared to LCR rats. Along with the increased fear conditioning described above, we also found that HCR rats showed lower locomotor activity in the activity box and less social interaction with a novel conspecific compared to LCR rats, behaviors that are associated with a more anxiotypic phenotype. These findings are consistent with a previous report showing that female HCR rats have significantly lower levels of locomotion and rearing in a 60-min novel environment test (but no differences in grooming behavior) compared to female LCR rats (Waters et al., 2010). Additionally, while HCR and LCR rats perform similarly on the elevated-plus maze (EPM) at a baseline, following a 1 hr period of restraint stress, HCR rats displayed more anxiety-like behavior. Additionally, EPM exposure following restraint stress elicited a significant increase in plasma corticosterone in HCRs but not LCRs. Administration of dexamethasone, a potent glucocorticoid, resulted in a normal suppression of plasma
corticosterone in both HCR and LCR rats, suggesting that negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis is similar in both groups. Another study found no locomotor activity differences in male HCR and LCR rats in a novel environment test (Burghardt et al., 2011). However, male HCR rats did show greater anxiotypic behavior in a light/dark box task as well as greater plasma corticosterone responses to the light/dark box. Together these findings suggest that high aerobic capacity rats also have a somewhat higher anxiotypic profile than low capacity rats, and this difference in stress responsivity might have broad influences on other behaviors, including cognitive function.

The link among exercise, neurogenesis and stress has been hypothesized to be a buffering of neural progenitor cells via an increase in inhibitory GABAergic interneurons in the hippocampus and the regulation of glucocorticoid receptors (Schoenfeld and Gould, 2013, Schoenfeld et al., 2013). While we found no significant differences in GR or MR in the hippocampus, HCR rats do appear to have a higher ratio of GR:MR, which could support a hippocampus that is buffered against the cytotoxic effects of corticoistrone but also highly sensitive to small changes in corticosterone levels. A previous study also found no differences in GR and MR between male HCR and LCR rats (Burghardt et al., 2011), although it does not appear that the ratio of the two receptors is different by their analysis of in situ hybridization throughout the subregions of the hippocampus. Instead, Burghardt and colleagues (2011) found that HCRs had increased adrenal and decreased thymus weights as well as higher levels of corticotropin-releasing hormone (CRH) in the central nucleus of the amygdala, and exhibit greater contextual conditioning to cat odor and greater anxiotypic behavior in a light/dark task that is related to corticosterone responses. These behavioral findings mirror our observations of increased contextual fear generalization and anxiotypic behavior on activity and social
measures in HCR rats compared to LCR rats. Taken together, it seems that HCR rats have a stress regulation system that is highly responsive to stress or novelty, which arguably gives HCR rats an advantage over LCR rats in responding to potentially dangerous, salient signals or performing cognitive tasks.

We found no differences in the hippocampal expression of growth factors or steroid hormone receptors, suggesting that these factors do not play a role in the effect of aerobic capacity on hippocampal neurogenesis. A previous study also demonstrated that BDNF mRNA is no different between male HCR and LCR rats, as well as Sprague Dawley rats at a baseline, although its expression is enhanced similarly in all rats following voluntary wheel running (Groves-Chapman et al., 2011). It therefore seems possible that, while exercise experience increases the expression of growth factors that are thought to contribute to enhanced neurogenesis, aerobic capacity must increase neurogenesis by other mechanisms that might work in combination with growth factors under exercised conditions. For example, exercise-induced growth factors might particularly target cell proliferation, while aerobic capacity seems to be specific for neuronal survival and maturation. Differences in the stress regulation system or observations that LCR and HCR rats have differences in antioxidant and oxidative DNA damage-repairing systems (Sarga et al., 2013) could be responsible for the direct role of aerobic capacity on the hippocampus.

Taken together, these data suggest that aerobic capacity, whether inherent or acquired by exercise, is critical for an elevation in hippocampal neurogenesis and pattern separation ability. The HCR/LCR and LRT/HRT rats strains provide elegant models for isolating large differences in aerobic capacity and disambiguating the potentially confounding effects of exercise. The pattern separation tasks used in this study were designed to test hippocampal neurogenesis-dependent cognition without requiring the
choices to be motivated by fear or food deprivation, or responses that require physical abilities like running or swimming, which are likely affected by the selection for aerobic fitness traits. Our findings demonstrate a positive relationship among elevated aerobic capacity, increased neurogenesis, and accurate pattern separation ability. The profound effect of aerobic capacity on brain and behavior observed in these studies calls for the measurement of changes in aerobic capacity in future studies of exercise on the brain.
Chapter 3: Inherent and exercised-induced aerobic capacity are able to protect against chemotherapy-induced decline in cardiorespiratory and hippocampal fitness

Many patients experience progressive mild cognitive impairment (MCI) following treatments for cancer – a phenomenon commonly referred to as "chemobrain" (Nelson et al., 2007). Chemotherapy induces MCI in about 10-40% of breast cancer patients, and is frequently described as a deficit in working memory and lack of concentration (Matsuda et al., 2005). Aerobic exercise has been shown to be particularly beneficial to these domains of cognition and can mitigate cognitive decline due to aging (Kramer et al., 2006, Voss et al., 2011) and age-associated neuropathologies such as MCI, Alzheimer's, and vascular dementia (Laurin et al., 2001, Baker et al., 2010, Foster et al., 2011). Studies that specifically examine the potential for exercise to benefit neurocognition in cancer patients are lacking.

In rodents, voluntary wheel running has been shown to protect against a decline in spatial working memory induced by 5-fluorouracil/oxaliplatin chemotherapy (Fardell et al., 2012) and by whole-brain irradiation (Wong-Goodrich et al., 2010). In these studies, the decline in hippocampal neurogenesis induced by these cancer treatments and the exercise-induced enhancement of neurogenesis is hypothesized to be the mechanism by which this hippocampal-dependent cognitive ability is impaired and improved, respectively. Chemotherapy and radiation therapy are cytotoxic not just to cancer cells but also normal dividing cells (Blagosklonny and Pardee, 2001), such as those proliferating in the neurogenic niches of the brain. While the direct impact on neurogenesis might be central to chemotherapy-induced cognitive impairment, other effects on the blood-brain barrier (BBB), oxidative stress, the HPA axis, and
neuroinflammation, for instance, might also be crucial or occur in combination to impair a wide-range of cognitive abilities (Seigers and Fardell, 2011).

Beyond the context of chemotherapy, the link between aerobic exercise, hippocampal neurogenesis, and cognition is well-established (van Praag, 2008). However, exercise is a broadly-used term and can include protocols that range from physical activity (e.g., voluntary wheel running) to endurance exercise training (e.g., incremental treadmill running). While aerobic exercises are thought to strengthen cardiorespiratory fitness (CRF) by increasing aerobic capacity, or the body's ability to use oxygen (Gaesser and Rich, 1984), the vast majority of studies on exercise do not measure changes in aerobic capacity (e.g., via direct VO$_2$ max measurements or estimations using a fitness test, such as on a treadmill or cycle ergometer).

The American Heart Association has recently called for the assembly of a national registry of CRF across a large and diverse population, given that the (relatively limited) data on CRF suggests that it is one of the most powerful predictors of cardiovascular and all-cause mortality (Kaminsky et al., 2013) as well as cancer survival (Jones et al., 2012a, Jones et al., 2012b). For instance, even a small increase in aerobic capacity (e.g., 3.5 mL O$_2$/kg-min or 1 metabolic equivalent, "MET") is associated with an 12-18% reduction in cardiovascular mortality in healthy men (Myers et al., 2002) and women (Barlow et al., 2012). Female breast cancer patients have an impairment in aerobic capacity that is related to their breast cancer treatment status (Peel et al., 2014). This decrement in aerobic capacity is seen even 7 years after treatment when compared to age-matched healthy controls (Lakoski et al., 2013, Peel et al., 2014). Endurance exercise training has been shown to be an effective intervention that improves CRF in addition to quality of life, physical functioning, and fatigue levels in breast cancer patients (McNeely et al., 2006). Whether or not an increase in aerobic capacity plays a causal role in these
physical benefits of exercise in cancer patients has not yet been examined. Furthermore, the role of aerobic capacity in a cognitive benefit of exercise following chemotherapy or providing protection prior to treatment is also largely unknown, although it seems likely considering the relationships among chemotherapy-induced cognitive impairment, aerobic exercise, and neurogenesis.

While measurements of aerobic capacity are largely absent from the literature of the effects of exercise on the brain, a few studies have demonstrated a relationship between aerobic capacity and cognitive behavior in humans. For instance, 10 weeks of high-intensity aerobic exercise was associated with the greatest increase in performance on a cognitive flexibility task in healthy adults, compared to minimal or moderate exercise conditions, and this enhanced cognition was positively correlated with the degree to which VO$_2$ max had increased (Masley et al., 2009). Consistent with the idea that intense aerobic exercise regimens aimed at increasing VO$_2$ max might be neuroprotective, another study found that 6 months of high-intensity exercise, compared to stretching, improves performance on a battery of cognitive tasks in patients with MCI (Baker et al., 2010). To further highlight a specific role for aerobic capacity, baseline VO$_2$ max – but not self-reported physical activity – was able to predict the preservation of cognition in older adults 6 years later (Barnes et al., 2003). Additionally, young adults who significantly increased their VO$_2$ max following a 6-week high-intensity aerobic exercise regimen (or high responders) were more accurate on a visual pattern separation task compared to low responders, and change in aerobic capacity was positively correlated with change in performance on this cognitive task, which is thought to be dependent on levels of hippocampal neurogenesis (Déry et al., 2013). In contrast, adults who scored high on the Beck Depression Inventory (BDI), who are hypothesized to have lower levels of neurogenesis, performed significantly worse than those with relatively low
BDI scores on this pattern separation task (Déry et al., 2013). The current study sought to investigate whether changes in the brain, such as neurogenesis, might underlie the potential for increased aerobic capacity, rather than exercise per se, to be neuroprotective.

To isolate aerobic capacity as a component of exercise that might be crucial for protection from cancer treatments independent of potential confounds that might come with exercise (e.g., stress, muscle soreness, exhaustion), we took advantage of rats selectively bred for high (HCR) and low (LCR) aerobic capacity for running (Koch and Britton, 2001). Selective breeding was based on rats’ treadmill running performance on a fitness test from a heterogeneous N:NIH stock founder population. By the 6th generation of selection, untrained HCR and LCR rats differed by 170% in aerobic capacity (Koch and Britton, 2001). We recently reported that the 31st generation of HCR rats, compared to LCR rats, have a 227% enhancement in the number of young neurons in the adult hippocampus and an enhancement in hippocampal-dependent learning and memory (Chapter 2). The HCR/LCR rat model provides the opportunity to examine the extent to which an elevation in aerobic capacity might be the key factor of exercise training that is responsible for protecting the cardiovascular system as well as the hippocampus from chemotherapy-induced toxicity.

In the first experiment, we examined the effects of doxorubicin chemotherapy on cardiorespiratory fitness and hippocampal neurogenesis in female HCR and LCR rats. We chose female rats because doxorubicin is a standard chemotherapeutic agent used in treating breast cancer (Goldhirsch et al., 2005), which predominantly occurs in females [estimated 232,670 new female cases vs. 2,360 new male cases in the United States in 2014 (Siegel et al., 2014)]. Fitness tests (tests of aerobic capacity) were administered before, immediately after, and one month following 4 weekly injections of doxorubicin.
While doxorubicin treatment functions as a model of chemotherapy-induced MCI, it is also an oxidative assault on the cardiovascular system that allows us to investigate how an induced-reduction ("knockdown") of aerobic capacity might impact rats with initially high or low aerobic capacity. We hypothesized that inherent high aerobic capacity, which is accompanied by inherent high levels of neuronal survival, might protect against the chemotherapy-induced assault on the cardiovascular and nervous systems.

In the second experiment, we investigated the potential for exercise training to rescue deficits in CRF and neurogenesis induced by an inherent low aerobic capacity phenotype or doxorubicin chemotherapy. Female HCR and LCR rats were given weekly injections of doxorubicin for 4 weeks while undergoing an individualized high intensity interval training regimen. Fitness tests were administered before and after doxorubicin treatment. The goal of these studies was to elucidate whether aerobic capacity is key factor for exercise to provide protection against a chemotherapy-induced impairment in CRF and hippocampal neurogenesis.

**Methods**

**Animals**

Female rats selectively bred for low (LCR) or high (HCR) aerobic capacity for treadmill running (Koch and Britton, 2001) from the 28th and 31st generations were obtained from the Koch Britton Lab (University of Michigan, Ann Arbor, MI). Rats were transported to the Duke University vivarium, where they were double-housed in ventilated shoebox cages in a climate controlled room with standard chow and water *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee of Duke University.
Experiment 1: Effect of doxorubicin treatment on HCR and LCR rats

HCR (n = 10) and LCR (n = 7) rats (12-14 months-old) of the 28th generation of the selection process were used in this study. Consistent with the findings of Wisloff et al. (2005), the body weights (± SEM) of the LCR rats (207 ± 5.70 g) were significantly greater than those of the HCR (163 ± 3.05 g) rats (p < 0.05). Rats were kept on a 12 hr light-dark cycle (lights on at 07:00), in which all behavioral handling within the light phase. Prior to initial fitness testing, all rats received daily acclimation to a standard rodent treadmill for one week as previously described (Koch and Britton, 2001): rats began with the treadmill set at a low speed of 2 m/min at a 15° incline, and every 2 minutes, the speed was increased by 2 m/min until 12 min had passed. Rats were then returned to their home cage. Following acclimation, an initial fitness test (fit test) was conducted to evaluate maximal aerobic capacity, an estimate of maximal oxygen consumption (VO₂ max). Rats were fit tested using the same protocol as acclimation except the rats were manually encouraged to continue running past 12 min until they met our criterion for exhaustion, defined as 3 occurrences of refusing to run even when guided by the experimenter’s hand. Rats were tested in this fashion for 2 consecutive days, and the maximal distance (m) run on either trial was used as a measure of aerobic capacity. Rats were then divided into experimental conditions that received either doxorubicin (LCR-dox, n = 3; HCR-dox, n = 6) or the saline vehicle (LCR-sal, n = 4; HCR-sal, n = 4). Doxorubicin hydrochloride (4 mg/kg body weight in saline) or an equivalent volume of saline was administered intravenously through the tail vein to isoflurane-anesthetized rats once a week for 4 weeks. We chose this dose level and treatment regimen because it is similar to what has been used in previous rodent research (Christie et al., 2012) and what has been provided to patients with advanced breast cancer (Cobleigh et al., 1999). One HCR rat and 2 LCR rats became ill (likely
congestive heart failure as determined by the staff veterinarian) and were sacrificed prior to the end of the experiment; therefore, their data was not included in any analysis. Approximately 1 week and 1 month following the last injection, rats were given a midpoint and final fit test, respectively. For 3-day periods throughout the experiment, each rat was vaginally swabbed to examine stage of estrous cycling; the majority of rats were found to be showing the vaginal mucosal variations associated with the estrous cycle, even at the end of the experiment. Two days following the final fit tests, all rats were euthanized with CO₂ and brains were rapidly dissected. The right and left hemispheres were post-fixed in 4% paraformaldehyde for histological procedures. Coronal sections (60 µm) containing the left dorsal hippocampus underwent immunohistochemistry for DCX (1:300 goat polyclonal, SCBT), Ki67 (1:200 rabbit polyclonal Abcam), or Iba1 (1:1000 rabbit polyclonal, Wako).

**Experiment 2: Effects of doxorubicin and exercise training on HCR and LCR rats**

HCR (n = 22) and LCR (n = 22) rats (10-12 months-old) of the 31st generation of the selection process were used in this study. The body weights of the LCR rats (290 ± 6.22 g) were significantly greater than those of the HCR (232 ± 4.02 g) rats (p < 0.0001). Rats were kept on a reversed 12 hr light-dark cycle (lights off at 09:00), in which all behavioral handling occurred within the dark phase, the normal active period for nocturnal species like rats, under dim lighting. All rats were acclimated to a standard rodent treadmill and underwent 2 initial fitness test trials to evaluate maximal aerobic capacity as described above and see: (Koch and Britton, 2001). Rats then received a tail vein (i.v.) administration of doxorubicin hydrochloride (dox; 4 mg/kg body weight of 2 mg/ml solution followed by 0.2 ml saline flush) or an equivalent volume of saline once a week for 4 weeks. Following a 24-48 h recovery period from each injection, rats either
underwent 5 consecutive days of individualized treadmill endurance training (trained) or were handled in the room during the times other rats were running (untrained). The training procedure occurred on a rodent treadmill at a 25° incline and consisted of a 15 min warm-up at 40-50% maximal aerobic capacity, followed by alternating intervals of 8 minutes at 85-90% and 2 minutes at 50-60% until a criterion for exhaustion was met (3 occurrences of refusing to run even when guided by the experimenter’s hand) or until 60 minutes had elapsed.

The experimental groups were divided as following: LCR-sal-untrained (n = 5), LCR-sal-trained (n = 5), LCR-dox-untrained (n = 6), LCR-dox-trained (n = 6), HCR-sal-untrained (n = 5), HCR-sal-trained (n = 5), HCR-dox-untrained (n = 6), HCR-dox-trained (n = 6). All rats received 2 final fitness test trials to re-evaluate AC. To measure corticosterone metabolites (CORT), fecal samples were collected during the first final fitness test trial ("baseline" timepoint), 7-9 hours after the trial ("fit test" timepoint), and during the second fit test trial the following day ("recovery" timepoint). Fecal samples were also 31-34 hrs ("circadian" control timepoint) after the second fitness test trial to evaluate the corticoisterone response during time-of-day that fitness tests had been conducted. Fecal samples were either collected from each rat’s treadmill lane during the fit tests or after being placed in an individual cage for 3 hours. Fecal samples were collected at the times listed because plasma levels of corticosterone are reflected in fecal metabolites in approximately 7-9 hrs after corticosterone is released into blood (Thanos et al., 2009).Within a week following the final fitness test, 2-3 rats from each group were anesthetized to receive magnetic resonance imaging (MRI) of the heart and brain.

Within 9 days following the final fit tests, all rats were euthanized with CO₂ and brains were rapidly dissected. The right hemisphere was post-fixed in 4% paraformaldehyde for histological procedures and the left hemisphere was flash frozen in
isopentanol over dry ice and stored at −80º for molecular processing. Coronal sections (60 µm) containing the right dorsal hippocampus underwent immunohistochemistry for the young neuronal marker DCX (1:300 goat polyclonal, SCBT) as described previously (Chapter 2). Fecal samples were lyophilized, sifted, weighed, and reconstituted in 80% methanol at 200 mg/ml. Samples were centrifuged and the supernatant was stored at −80º. Fecal CORT was analyzed using a Corticosterone ELISA kit (Enzo Life Sciences, Farmingdale, NY) as per the manual’s instructions using a 1:40 sample dilution.

Results: Experiment 1

**HCR rats have higher aerobic capacity than LCR rats, even following chemotherapy**

As expected, 12-14 month-old HCR rats showed a 316% higher (p < 0.0001) aerobic capacity than LCR rats on an initial treadmill fit test (Fig. 3.1A). One week following 4 weekly injections of doxorubicin or saline vehicle, HCR and LCR rats were given a midpoint fitness test, in which there was a significant main effect of rat strain (F<sub>3,13</sub> = 5.89, p < 0.01) but not a main effect of treatment or an interaction between treatment and string. On a final fitness test given one month later, however, there was a significant main effect of treatment (F<sub>3,13</sub> = 7.23, p < 0.05) in addition to a main effect of strain (p < 0.01) with no significant interaction. Post hoc analysis revealed that HCR-sal rats ran significantly greater final distances than HCR-dox (p < 0.05), LCR-sal (p < 0.01) and LCR-dox rats (p < 0.01). While post hoc tests did not show that HCR-dox rats had significantly different final distances from LCR-sal or LCR-dox rats, they did appear to have higher aerobic capacity by direct comparisons (Student’s t-test, HCR-dox vs. LCR-dox, p < 0.01; HCR-dox vs. LCR-sal, p < 0.05). Overall, doxorubicin produced a robust, 50% decrease in aerobic capacity in HCR rats and a milder, 25% decrease in LCR rats.
Figure 3.1: Inherent high aerobic capacity protects rats from chemotherapy-induced decline in neurogenesis. A) Prior to saline (sal) or doxorubicin (dox) injections (Initial), HCR had a 3 times greater maximal aerobic capacity (m) than LCR rats ($p < 0.0001$). Following dox injections, there was a significant decrease in running distance for HCR rats ($p < 0.001$), but not for LCR rats. HCR rats treated with dox still ran a greater distance than LCR rats treated with SAL ($p < 0.001$). *main effect of strain ($p < 0.001$); *main effect of dox ($p < 0.05$). B) Number of DCX+ young neurons in the dentate gyrus (DG) at the time of sacrifice. *main effects of strain ($p < 0.001$); *main effect of dox ($p < 0.01$). In addition, HCR rats treated with DOX were not significantly different than LCR rats treated with SAL. C) Number of Ki67+ proliferating cells in the DG.
**HCR rats are protected against a doxorubicin-induced decline in neurogenesis**

At the time of sacrifice, HCR rats had significantly more DCX+ young neurons in the dentate gyrus of the hippocampus compared to LCR rats (main effect of strain, \( F_{3,13} = 14.59, p < 0.0001 \)) (Fig. 3.1B). Doxorubicin significantly reduced this neurogenesis in both LCR and HCR rats (main effect of treatment, \( p < 0.01 \)); however, unlike the effects on aerobic capacity, doxorubicin had caused a more robust decrease in the number of DCX+ neurons in LCR rats (78% decrease) compared to HCR rats (42% decrease). There was no significant interaction of strain and injection. There were also no significant differences in the volume of dentate gyrus from which counts were made among groups. Post hoc analysis showed that HCR-sal rats had more DCX+ cells than HCR-dox (\( p < 0.05 \)), LCR-sal (\( p < 0.01 \)), and LCR-dox (\( p < 0.001 \)) rats. HCR-dox rats had significantly more DCX+ cells than LCR-dox (\( p < 0.05 \)) rats and were no different from LCR-sal rats.

There were no significant differences in the number of Ki67+ proliferating cells in the dentate gyrus among all groups (\( F_{3,13} = 0.63, \text{NS} \)) (Fig. 3.1C). Taken together, doxorubicin resulted in a negative effect on the survival of young neurons, but not the rate in their proliferation, in the hippocampus one month following the halt of treatment, and HCRs were partially protected from this decline.

**HCR rats have fewer quiescent microglia than LCR rats**

To assess whether inherent aerobic capacity or chemotherapy cause differences in the activation state of microglia, we categorized Iba1+ cells into four types (thin, thick, stout, or amoeboid) based on their shape, size, and morphology in the dentate gyrus and ranging from a quiescent to activated microglia state (Fig. 3.2B). Due to differences in the volumes assessed, microglia counts are represented as estimated number over dorsal DG volume (a density measure). HCR rats expressed significant fewer thin microglia
compared to LCR rats ($F_{3,13} = 1.98, p < 0.05$) (Fig 3.2A). There were no significant differences in the number of thick, stout, or amoeboid microglia.

**Figure 3.2:** HCR rats have fewer quiescent microglia than LCR rats regardless of doxorubicin treatment. **A** Iba+ microglia were classified into groups based on their morphology and level of activation. Data is presented as a density measure (number of cells/DG volume). *HCR rats have significantly fewer thin microglia than LCR rats ($p < 0.05$). **B** Photomicrograph representations of thin, thick, stout, and amoeboid microglia in order from quiescent to activated morphology.
Results: Experiment 2

Training improves aerobic capacity in LCR and HCR rats and protects against doxorubicin

To evaluate the potential for exercise training to protect against the doxorubicin-induced decline in aerobic capacity and hippocampal neurogenesis rats that vary in inherent aerobic capacity, LCR and HCR rats were trained using a high intensity interval training protocol 5 days/week following each of 4 weekly doxorubicin or saline injections, or remained as sedentary controls. Consistent with Experiment 1, HCR rats had a 211% higher aerobic capacity than LCR rats during initial treadmill tests of aerobic capacity ($p < 0.0001$) (Fig. 3.3A). There were no significant differences in initial fit test results within LCR or HCR experimental groups that were later divided to test the effects of doxorubicin treatment and exercise training. Also similar to Experiment 2, doxorubicin treatment led to an impairment in aerobic capacity for HCR rats ($F_{3,18} = 6.39$, $p < 0.01$, main effect of treatment, $p < 0.05$) but not for LCR rats ($F_{3,18} = 7.53$, $p < 0.01$, main effect of treatment, NS) as measured by the final fit test given after the final week of training (Fig. 3.3B). We found that exercise training improved aerobic capacity for LCR ($F_{3,18} = 7.53$, $p < 0.01$, main effect of training, $p < 0.001$) and HCR ($F_{3,18} = 6.39$, $p < 0.01$, main effect of training, $p < 0.01$) rats regardless of dox treatment (no significant training*dox interactions). Analysis across all LCR and HCR groups ($F_{7,36} = 13.11$, $p < 0.0001$) revealed main effects of rat strain ($p < 0.0001$), training ($p < 0.0001$), and doxorubicin treatment ($p < 0.05$) with no significant effect interactions. Post hoc analysis (Tukey HSD) revealed no significant differences between HCR-sal-trained and HCR-dox-trained rats, indicating that exercise training prevented the dox-induced decline in aerobic capacity in HCR rats.
Figure 3.3: Training provides protection against chemotherapy in HCR but not LCR rats. A) During the initial fit tests, HCR rats ran approximately 2.3 times more than LCR rats. *p < 0.0001. B) During the final fit tests, dox treatment significantly lowered the aerobic capacity for HCR but not LCR rats, while training increased aerobic capacity for all conditions. Levels not connected by the same letter are significantly different. C) Linear representation of aerobic capacity at the initial and final fit tests. D) Number of DCX+ young neurons in the dentate gyrus. Levels not connected by the same letter are significantly different. E) Significant correlation between aerobic capacity and number of DCX+ neurons (p < 0.001).
Effects of training and doxorubicin on neurogenesis LCR and HCR rats

Consistent with the selection for aerobic capacity, saline-treated rats displayed a 127% higher aerobic capacity than LCR rats ($F_{3,33} = 5.44, p < 0.001$, main effect of strain, $p < 0.0001$) (Fig. 3.3D). Consistent with our previous finding in Experiment 1, dox had a main effect on the number of DCX+ cells in LCR but not HCR rats (main effect of dox, $p < 0.05$). Training enhanced neurogenesis across all groups (main effect of training, $p < 0.01$), although only significant (Tukey Post Hoc tests) in saline-, but not dox-, treated LCR and HCR rats. This pattern was similar but somewhat distinct from the broad effect training had on performance on the final fit tests (Fig. 3.3B).

Differences in stress responsivity between HCR and LCR rats

The presence of CORT (corticosterone metabolites) was assayed from fecal samples collected at timepoints to indicate the stress response before (baseline), during the first trial of the final fit test (fit test 1), after (recovery), during the second trial of the final fit test the following day (fit test 2), after (recovery 2), and for a timepoint the following day when no fit test was given (circadian control) (Fig. 3.4). Across all timepoints, there were no significant fluctuations in CORT in LCR-sedentary-sal rats ($F_{5,22} = 0.56, NS$), LCR-sedentary-dox rats ($F_{5,24} = 2.28, NS$), HCR-sedentary-sal rats ($F_{5,22} = 1.02, NS$), HCR-trained-sal rats ($F_{5,22} = 2.15, NS$), or HCR-trained-dox rats ($F_{5,22} = 1.34, NS$). There were significant differences in the CORT response of LCR-trained-sal rats ($F_{5,24} = 4.97, p < 0.01$), LCR-trained-dox rats ($F_{5,30} = 6.16, p < 0.001$), and HCR-sedentary-sal rats ($F_{5,30} = 3.57, p < 0.05$). Across these groups, there appeared to be a peak in CORT from the fit test 1 timepoint (significance level of A, compared to other levels of AB or B), indicating a heightened response to that stressor.
Figure 3.4: Differential CORT responses to an aerobic fitness test.
Fecal CORT response and recovery timepoints to the two final fit test trials for HCR and LCR rats given saline (sal) or doxorubicin (dox) and kept sedentary (sed) or trained. *Significantly elevated level of CORT indicated by one-way ANOVA, $p < 0.05$.

Discussion

Using rats selectively bred for inherent differences in aerobic capacity, this study provides strong evidence that aerobic capacity, a measure of cardiorespiratory fitness, is causally linked to the protection of the cardiovascular system and the brain. The present studies demonstrate that doxorubicin, a chemotherapeutic agent commonly used for the treatment of breast cancer, has a severe and progressive impact on aerobic capacity and hippocampal neurogenesis. Rats with high baseline levels of aerobic capacity (HCR) were buffered from deterioration of cardiorespiratory fitness and the loss of neurogenesis such that, one month following doxorubicin treatment, they had higher aerobic capacity and more new neurons than rats with inherently low aerobic capacity (LCR). Rats that engaged in endurance exercise training during the period of doxorubicin treatment, regardless of HCR or LCR phenotype, displayed an enhancement in aerobic capacity. Remarkably, endurance training completely rescued the drastic doxorubicin-induced...
reduction in aerobic capacity that occurred in HCR rats. Additionally, this training protected the hippocampus from doxorubicin-induced decline in neurogenesis, particularly in HCR rats. Taken together, these data reveal that both inherently high aerobic capacity and increased aerobic capacity acquired via exercise training are able to both prevent and protect against chemotherapy-induced cardiovascular and hippocampal decline. These findings have important implications for the role of aerobic capacity in the mechanism underlying how exercise ameliorates cardiovascular disease and cognitive impairments in cancer patients.

The finding that HCR rats, compared to LCR rats, have 2- to 3-fold higher aerobic capacity as well as 2- to 3-fold higher levels of neurogenesis in the hippocampus under baseline conditions is consistent with our previous findings (Chapter 2). The differences in neurogenesis between HCR and LCR rats were again observed specifically in the number of young neurons present in the hippocampus and not in the number of proliferating cells. Perhaps one of the reasons that higher aerobic capacity is so effective at ameliorating the deficits due to doxorubicin is that doxorubicin causes a deficit in neuronal maturation and not in the survival of proliferating cells (Christie et al., 2012). While it is possible that hippocampal cell proliferation might have been disrupted around the time of doxorubicin administration, its long-term effects clearly resulted in a sustained deficit in young neuronal survival. In contrast, we saw no effects of this treatment on cell proliferation one month following the cessation of doxorubicin treatment.

Higher levels of neurogenesis in HCR rats were associated with a lower level of quiescent microglia in the DG. This finding is consistent with previous reports that upregulated neurogenesis may stimulate an activated microglia response in the dentate gyrus, resulting in a decrease in the number of resting, quiescent microglia (Sierra et al.,
The finding that microglia state was not significantly affected by prior doxorubicin treatment suggests that doxorubicin does not have an enduring effect on immune system inflammation in the hippocampus. Methotrexate chemotherapy has been shown to activate microglia 3 weeks following treatment, but this activation was not associated with markers of neuroinflammation, such as cytokine levels, or observed impairments in cognition (Seigers et al., 2010). Taken together, microglia play a likely role in regulating or responding to levels of neurogenesis that are modified by alterations in aerobic capacity, but may not have a significant involvement in the long-term consequences of chemotherapy on hippocampal function.

Chemotherapy may also alter the blood-brain barrier (BBB), oxidative stress, or the HPA axis, and these factors may lead to changes in adult neurogenesis (Seigers and Fardell, 2011). Additionally, chemotherapeutic agents, such as doxorubicin, have been described as having complex and varied effects on cellular processes, including the inhibition of DNA synthesis and the generation of free radicals (Konat et al., 2008). Because doxorubicin does not easily cross the BBB, it has been proposed that it impacts the brain indirectly by introducing oxidative stress (Seigers and Fardell, 2011). In support of this idea, administration of the antioxidant N-acetyl cysteine (NAC) throughout the course of doxorubicin treatment has been shown to prevent an impairment in hippocampal memory function as measured by a passive avoidance test in rats (Konat et al., 2008). Alternatively, it is possible that doxorubicin can penetrate the BBB if it has been compromised (Bigotte and Olsson, 1983), perhaps due to more long-term effects of cytotoxic chemotherapy, or at sites surrounding neurogenic, or vascular, niches of the brain that possess unique properties (Tavazoie et al., 2008). The later would be particularly relevant for the effects of chemotherapy on hippocampal neurogenesis.
Somewhat surprising, HCR rats exhibited a significant fecal corticosterone response following a fitness test in contrast to LCR rats. This finding is consistent with a previous reports that HCR rats, but not LCR rats, show more anxiety-like behavior on the elevated-plus maze and elicit a significant increase in plasma corticosterone following a 1 hr period of restraint test (Waters et al., 2010). Administration of dexamethasone, a potent glucocorticoid, resulted in a normal suppression of plasma corticosterone, suggesting that both HCR and LCR rats have similar negative feedback of the HPA axis and other factors, such as higher levels of CRH in the amygdala of HCR rats, might be at play in regulating the stress response. Interestingly, we observed that following a period of endurance exercise training, HCR rats no longer displayed a robust corticosterone response to a fitness test. In contrast, trained LCR rats manifested a corticosterone response to a fitness test that coincided with their increased aerobic capacity. Prior doxorubicin treatment had no significant effect on corticosterone. These influences of inherent aerobic capacity strain and endurance training parallel the results a study also showing a significant interaction between HCR/LCR strain and running wheel exercise on plasma corticosterone response to running wheel access (Waters et al., 2008). Taken together, exercise training seems to induce a stress response in LCR rats that does not occur under sedentary conditions, perhaps due to the short time or distance LCR rats are able to run at a baseline. Surprisingly, exercise training in HCR rats mitigates a stress response to running, despite running significantly longer distances than at sedentary conditions, which were already at a high aerobic capacity level. Prior-doxorubicin treatment had no apparent effect on these corticosterone responses. Regulation of the stress response has important implications for how neurogenesis, anxiety-like and depressive-like behavior, and cognition, are modulated by exercise, which have all previously been related (Schoenfeld and Gould, 2012).
In the current studies, the use of doxorubicin could be considered as an oxidative assault on aerobic capacity, which we have shown to be directly correlated with neurogenesis levels. This manipulation was able to knockdown high levels of aerobic capacity and neurogenesis in HCR rats to levels similar to those exhibited by LCR rats. Endurance exercise training that occurred throughout doxorubicin administration was able rescue these deficits such that doxorubicin-treated HCR rats that exercised displayed aerobic capacity and neurogenesis levels no different from saline-treated HCR rats. Because levels of neurogenesis are thought to influence many cognitive processes and have been hypothesized to underlie chemotherapy-induced effects on cognition, alterations in aerobic capacity may be the mechanism by which cognition is damaged by chemotherapy and, in turn, rejuvenated by exercise.

Taken together, our results point to a causal link between cardiorespiratory fitness, hippocampal neurogenesis, and chemotherapy-induced cognitive impairment. These findings may offer an explanation for clinical observations of idiosyncratic vulnerability to the cognitive impairments of “chemobrain,” which may depend on the extent to which aerobic capacity is impacted. Findings from the HERITAGE Family Study have revealed that human populations have significant variability in inherent CRF as well as in the capacity to show increased CRF following a period of exercise training (Bouchard et al., 1998, Bouchard et al., 1999, Bouchard et al., 2000). Therefore, like HCR and LCR rats, people might be at "low" or "high" risk prior to receiving chemotherapy or in their physical and neurocognitive responses to the treatment. Our findings provide support for the view that increased aerobic capacity may function as a putative therapy against the cytotoxic effects of chemotherapy, regardless of whether it is gained from an inherent disposition or endurance exercise training.
Conclusions

The current body of work isolated aerobic capacity as a component of physical fitness that appears to be crucial for exercise to enhance neurogenesis and pattern separation ability. A novel method was designed to evaluate neurogenesis-dependent pattern separation ability in rats that differ in physical fitness. This task, utilizing parametric alterations in plastic object configurations, is sensitive to an irradiation-induced reduction of hippocampal neurogenesis as well as increases in neurogenesis evident in rats with high levels of aerobic capacity, whether inherent or acquired by exercise. A high level of aerobic capacity also protects against a sever assault on aerobic capacity and neurogenesis induced by doxorubicin chemotherapy. Exercise training is able to restore aerobic capacity and hippocampal neurogenesis to high levels in rats of inherent high aerobic capacity, and appears to increase these measures even in inherent low aerobic capacity rats. Taken together, variations in aerobic capacity might underlie exercise's effects on brain via its modulation of hippocampal neurogenesis, particularly neuronal survival and maturation. These findings could have implications for the recommendation of physical exercise as a cognitive therapy in humans, which vary in inherent and acquired cardiorespiratory fitness. In addition to cardiorespiratory fitness, aerobic capacity is a critical component of the fitness of the hippocampus and should be emphasized as crucial variable in studies on the effects of exercise on the brain and cognition.
Appendix A: Voluntary Running Can Protect Against Whole-brain Irradiation-induced Neurocognitive Deficits Under Severe Immunodeficiency

Abstract

It is currently unclear whether an intact immune system is required for the maintenance of neurocognitive function or its protection by exercise. Previously, we have shown that voluntary exercise after whole-brain irradiation (WBI) prevents memory decline and enhances hippocampal neurogenesis in immunocompetent C57BL/6 mice. The current study investigated the potential for voluntary wheel running to prevent a WBI-induced decline in hippocampal function in NOD scid gamma (NSG) mice, which constitutently lack mature T and B cells, functional NK cells, and the IL-2R common gamma chain. Both sham-irradiated and irradiated NSG mice were able to learn and remember an escape location on the Barnes Maze one week following WBI. Daily exercise on a running wheel mitigated the deleterious effects of WBI on initial learning rate, reversal learning and memory one month post-WBI, the loss of DCX+ young neurons in the dentate gyrus of the hippocampus, and hippocampal levels of BDNF, VEGF, and microglia-specific Iba1. Running also mitigated a WBI-induced increase in pro-inflammatory cytokines TNF-α, IFN-γ, and IL-1β in the hippocampus and led to a potentially compensatory increase in anti-inflammatory cytokines IL-5 and IL-10. In conclusion, exercise is an effective strategy for improving hippocampal neurogenesis, learning, and memory following whole-brain irradiation even in immunodeficient mice.
Introduction

Whole-brain irradiation (WBI) is an established model of injury that halts cell division and produces progressive neurocognitive impairments. One therapy that appears to be effective in restoring adult hippocampal neurogenesis and mitigating memory decline in C57BL/6 mice following WBI is daily voluntary wheel running (Wong-Goodrich et al., 2010). Running also increases the hippocampal expression of trophic factors, such as vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) in irradiated mice. These findings suggest that exercise preserves neurocognitive function following WBI treatment by altering the hippocampal microenvironment, which includes upregulating growth factors that can enhance neurogenesis in mice with intact immune systems. However, it is not known if exercise can aid neurocognition if the immune system is compromised.

It is thought that the brain’s innate and adaptive immune systems regulate adult cell proliferation and neuronal survival, as well as the hippocampal response to enrichment or injury (Kohman and Rhodes, 2013). For example, the presence of CNS-specific T cells – but not B cells – appears to be required for adequate cell proliferation in the subgranular zone (SGZ) of the hippocampus and subventricular zone (SVZ), normal expression of hippocampal brain-derived neurotrophic factor (BDNF), accurate spatial learning and memory, and the capacity for the hippocampus to upregulate neurogenesis in response to environmental enrichment (Ziv et al., 2006). However, it is not known whether T cells are also necessary for the neuroprotective effects of voluntary exercise following a neurological assault such as WBI.

Other evidence points to a role for microglia in regulating adult hippocampal neurogenesis. For example, basal levels of neurogenesis are modified by microglia that survey the hippocampal microenvironment with ramified processes and remove
apoptotic new cells through phagocytosis (Sierra et al., 2010). Microglia also appear to have a role in regulating hippocampal neurogenesis in response to environmental enrichment, voluntary running, injury, or infection (Kohman and Rhodes, 2013). While the state of activation of microglia can be observed by the thickening and retraction of their branches, they are distinguished as anti- or pro-neurogenic depending on the secretion of molecules with pro- or anti-inflammatory action, respectively (Harry and Kraft, 2008, Ekdahl et al., 2009, Ransohoff and Perry, 2009). While neuroprotective microglia activation is associated with the expression of IGF-1 and class-II major histocompatibility complex (MHC-II), neurotoxic microglia activation is associated with the expression of pro-inflammatory cytokines (e.g., IL-1α, IL-1β, IL-6, IFN-γ, and TNF-α) and free radicals such as nitric oxide. These neurotoxic microglia appear to compromise the survival, rather than proliferation or differentiation, of newly formed neurons (Ekdahl et al., 2003, Monje et al., 2003), which is in contrast to the observed effects of CNS-specific T cells on proliferation (Ziv et al., 2006). These data are particularly interesting because whole-brain irradiation elevates levels of pro-inflammatory IL-6, IFN-γ, and TNF-α in the mouse hippocampus, and while daily voluntary running has a neuroprotective effect on neurogenesis months after WBI, it has no significant effect on the levels of these pro-inflammatory cytokines (Wong-Goodrich et al., 2010). Taken together, these data suggest that voluntary exercise may induce the expression of anti-inflammatory cytokines (such as IL-5 and IL-10) along with growth factors to enhance the survival of newborn neurons and counter the deleterious effects of WBI directly on dividing stem cells and the reactive expression of pro-inflammatory microglia.

To the best of our knowledge, the potential for exercise to protect against a neurological assault has not yet been evaluated in a model of immunodeficiency. As a
starting point in our investigation of the role of the immune system in exercise-induced neuroprotection, we examined the effects of voluntary wheel running on brain and behavioral measures of hippocampal plasticity following WBI in NOD scid gamma (NSG) mice, which have lifelong compound immunodeficiencies. NSG mice not only lack mature T and B lymphocytes, but also have a complete null mutation of the IL-2R gamma chain, which results in deficient cytokine receptor binding for interleukin (IL) 2, 4, 7, 9, 15 and the absence of functional natural killer (NK) cells, which require IL-15 signaling to develop (Shultz et al., 2005). While NSG mice have dendritic cells and macrophages, such as microglia, these cells are functionally immature because of the NOD genetic background. For instance, bone marrow-derived macrophages from these mice show defective IL-1 secretion in response to stimulation with lipopolysaccharide (Shultz et al., 1995). However, it is unclear whether microglia in the NSG mice are capable of producing other pro-inflammatory cytokines or anti-inflammatory cytokines and growth factors in response to irradiation or exercise. Neutrophils and monocytes compose most of the remaining immune cells in NSG mice, although they possess fewer of these cells than are typically found in C57BL/6 mice (Bugl et al., 2013). Endothelial cells, astrocytes, and neurons are other cells in the hippocampal microenvironment that should be capable of producing cytokines and growth factors in NSG mice. We hypothesized that, despite these immunodeficiencies, voluntary running could protect against WBI-induced neurocognitive decline in NSG mice similar to that already observed in intact immune mice.
Methods

Animals

Twenty-three female NOD scid IL-2 receptor gamma chain knockout (NOD.Cg-Prkdc<sup>scid</sup> IL-2rg<sup>tm1Wjl</sup>/SzJ or NSG) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) at 6 weeks of age. Upon arrival to the Duke University vivarium, they were socially housed, 3-5 per shoebox cage, with standard chow and water <i>ad libitum</i>. Whole-brain irradiation was administered when mice reached 9 weeks of age. We chose female mice because women experience more adverse cognitive symptoms than males following brain irradiation (Kadan-Lottick et al., 2010, Raber, 2010). Mice were kept on a reversed 12/12-hr light-dark cycle with lights off at 07:00 hr, and all behavioral training occurred during the dark phase of the cycle. Figure 1 presents the timeline of all experimental procedures. All procedures were approved by the Institutional Animal Care and Use Committee of Duke University.
**Figure A.1. Experimental timeline.** NSG mice were given whole-brain irradiation (IRR) or sham irradiation (SHAM) on day 1. Starting on day 4, mice either remained socially housed or were given daily access to an individual running wheel (IRR-RUN) during the dark phase of their light cycle. One week following WBI, all irradiated mice began behavioral training on the Barnes maze (BM1). Mice were habituated to the maze (H), trained for three days on one escape hole location (T), and given a probe test (P) 1 hr following the last training session. One month post-IRR, mice were retrained to a different escape hole location (BM2), given a 1 hr delay probe test, and sacrificed 19 days later.

### Whole-brain irradiation

All mice were anesthetized with 250 mg/kg tribromoethanol (i.p.) and treated with bilateral cranial irradiation using a 350-kV orthovoltage radiator while the body was shielded by a lead plate (IRR, n = 20) or not irradiated (SHAM, n = 3). IRR mice received a single dose of 5 Gy X-ray irradiation at a dose rate of 258 cGy/min; this dose impairs neurogenesis and hippocampal-dependent learning and memory in mice (Mizumatsu et al., 2003, Rola et al., 2004).

### Voluntary running

Following WBI or sham treatment, all mice remained socially housed in their home cage. A subset of these mice (IRR-RUN, n = 8) were given individual daily access
to a running wheel for the remainder of the experiment. This was done by removing mice from their home cage at the onset of the dark cycle and placing them individually into a new cage with food and water *ad libitum* and a low profile, wireless running wheel (Med Associates, 15.5 cm diameter) that recorded the number of wheel revolutions per day for each mouse (Wheel Manager, Med Associates, St Albans, VT). Because mice were temporarily removed from home or running cages for Barnes maze training, wheel access varied between 6 and 10 h/day (mean: 8.37 ± 0.25) and distance travelled varied between 3 and 8 km/day (mean: 5.87 ± 0.62, calculated by number of counts times the 34.24 cm circumference of the center running path on each wheel).

**Behavioral training**

IRR and IRR-RUN mice were temporarily removed from home or exercise cages for Barnes maze (BM) training. Mice were always trained in the same order with experimental conditions alternated. Behavioral measures were conducted one week (BM1) and again, one month (BM2) after WBI. Half of each group began BM1 on day 7 after WBI, while the second half was offset by three days to ensure behavioral training occurred during the middle of the dark cycle (not in the first or final hour).

The Barnes maze (Barnes, 1979), a 92 cm diameter black platform elevated 107 cm above the floor with 20 holes (5 cm diameter) equally spaced 2 cm from the perimeter, was located in a well-lit room with salient extra maze cues. A dark escape box (20 x 7 x 8 cm) with woodchip bedding could be attached to the maze under any of the holes. During training and testing, the maze was illuminated by a bright light and white noise was played loudly in the test room. The maze was lightly wiped down with 70% ethanol between trials to reduce odors from previous mice. Mice were first habituated to the maze for 3 trials (20 min inter-trial interval). During habituation, mice were placed
in the center of the platform, allowed to explore for up to 90 s, and then guided to an escape box that was never used for BMI or BM2 training.

For BM1 training, the escape box was located under the center hole of the northeast quadrant (each quadrant had 5 holes). Mice received 4 training trials per day for 3 days (90 s trial maximum; 20 min inter-trial interval), followed by one 30 s probe test (with no escape box) that occurred 1 hr after the last training trial. One month post-WBI, mice were re-trained (4 trials/day for 3 days) to find a different escape hole location (BM2), which was 180° from the escape location used for BM1. Mice were then given a memory probe test 1 hr following the last reversal training trial. After BM2 was complete, the mice were given a reminder training trial followed by a 90 s trial with a curtain around the maze to determine if mice were navigating using hippocampal-dependent spatial memory using extra maze cues.

Latency to locate the escape hole (learning) and total time spent in each quadrant on probe trials when no escape was possible (memory) were recorded with a computerized video tracker (HVS Image) for statistical analyses.

**Tissue Harvesting**

Two months after WBI, all mice were anesthetized with isoflurane and rapidly decapitated. This timepoint was used because our previous report demonstrated that two months is sufficient to observe irradiation-induced cognitive and neurological deficits in C57BL/6 mice (Wong-Goodrich et al., 2010). Brains were dissected on ice and midsagittally sectioned into left and right halves. The left hemisphere was post-fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) and cryoprotected in 30% sucrose in 0.1M PB for 24 hrs prior to being sliced coronally into 40 µm sections on a freezing microtome through the rostral-caudal extent of the hippocampus. Every fifth section was
collected in 0.1% sodium azide in 0.1M PB to yield five series of 8-10 sections. The hippocampus from the right hemisphere was dissected, flash frozen in 50/50 isopentane/ethanol over dry ice, and stored at −80°C.

**Immunohistochemistry**

Dorsal hippocampal sections were incubated in 50% methanol and 3% hydrogen peroxide for 1 hr, washed with 0.01M PBS, incubated in blocking reagent and then a primary antibody solution at room temperature overnight. The following primary antibodies were used: mouse anti-PCNA monoclonal (1:100, Santa Cruz Biotechnology, Dallas, TX) to label proliferating cells and goat anti-DCX polyclonal (1:300, Santa Cruz) to label young neurons. The PCNA protocol included an additional incubation in Mouse on Mouse blocking reagent (M.O.M. kit, Vector Laboratories, Burlingame, CA) to block endogenous mouse antibodies prior to primary incubation. Sections were rinsed and incubated in biotinylated anti-mouse (Vector) for the PCNA protocol or biotinylated horse anti-goat (1:200, Vector) for the DCX protocol. Sections were again rinsed with PBS, incubated in an avidin-biotinylated peroxidase complex solution (ABC, Vector), and treated for peroxidase detection with diaminobenzidine (DAB, Vector) for PCNA or Vector SG substrate (Vector) for DCX. Sections were rinsed, mounted onto Superfrost Plus Micro Slides (VWR International, Radnor, PA), counter-stained with cresyl violet, and coverslipped for immunopositive cell quantification.

**Quantification of PCNA+ and DCX+ cells**

Brain sections containing PCNA+ cells that label proliferating cells and DCX+ cells that label young neurons in the dorsal dentate gyrus were quantified using Stereo
Investigator (MBF Bioscience, Williston, VT). Contours were drawn around the region that encompassed the dorsal and ventral blades, including the granule cell layer and subgranular zone using a 40x objective lens on a Nikon light microscope. There were no significant differences in dentate gyrus volume obtained from these contours (one-way ANOVA, $F_{2,18} = 2.54, \text{NS}$). We used a modified fractionators principle to move exhaustively throughout each region for 5 sections per brain, using an optical dissector height of 20 µm with a 2 µm guard zone to avoid over-sampling between brain sections. Exhaustive counting was performed because the number of stained cells in WBI mice was too low to sample randomly within a section. Dorsal dentate gyrus volume estimates were generated using the optical fractionator and according to Cavalleri’s principle (Mouton, 2002). Data are represented as the number of cells per dorsal dentate gyrus in one hemisphere.

**ELISAs**

Hippocampal tissue was thawed and lysed using T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) as described by the manufacturer. Collected protein was measured using a Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions for brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-1 beta (IL-1β), interleukin-5 (IL-5), and interleukin-10 (IL-10).

A separate ELISA protocol was designed to detect ionized calcium binding adaptor molecule 1 (Iba1) protein. A Microtiter plate (Corning Inc., Corning, NY) was coated with 1 µg polyclonal Iba1 antibody (Wako Chem., Richmond, VA) in carbonate buffer (pH 9.6), covered with adhesive plastic and incubated at 4°C overnight. The plate
was washed twice with 200 µl PBS and blotted on a paper towel. To ensure remaining protein-binding sites on plate are blocked, 200 µl blocking buffer was put on the plate and incubated for 2 hr at room temperature. Samples (100 µl; or 0-500 pg of diluted Iba1 protein to construct a standard curve, Wako Chem., Richmond, VA) were added to each well, and the plate was incubated for 90 min at 37°C. Samples were removed and the plate washed twice with 200 µl PBS. For detection, 100 µl of detection antibody anti-rabbit PE (BD Biosciences, Franklin Lakes, NJ) was added to each well and incubated for 2 hr at room temperature. The plate was washed 4 times with 200 µl PBS, and fluorescence was detected using a BioTek plate reader (Winooski, VT).

**Statistical analysis**

All data are presented as means ± standard error. Using an α level of 0.05, data were analyzed using ANOVA, repeated measures ANOVA (RMANOVA), and *a priori* comparisons where appropriate with JMP Pro v10.0.2 (SAS Institute, Cary, NC). NS indicates a *p* value that was not significant.

Swabs of vaginal secretions were collected daily during Barnes maze training and for three days leading up to the day of sacrifice. Mice were cycling regularly. Because there were no significant correlations between estrous cycle status and Barnes maze performance or hippocampal measures, the estrous cycle status of mice was not included in further analysis.
Results

Running reduced WBI-induced learning deficits in NSG mice

In order to determine if immunodeficient mice that lack mature T and B cells, mature macrophages, functional natural killer (NK) cells, and cytokine receptor binding for interleukin (IL) 2, 4, 7, 9, 15 and 21 (Shultz et al., 2005) are capable of hippocampal-dependent learning and memory, we trained non-irradiated (SHAM) NSG mice to learn a location of a spatial location and remember it after a delay. SHAM NSG mice easily learned to find the escape hole on the Barnes maze as revealed by a significant decrease in latency across training trials (RMANOVA, main effect of day $F_{(2,1)} = 447.90, p < 0.05$) (Fig. A.2A). Analysis of matched pairs revealed that SHAM mice had significantly shorter latencies after the first day (Day 2 - Day 1, $p < 0.01$; Day 3 - Day 1, $p < 0.05$) and reached asymptotic performance by the third day of training (Day 3 - Day 2, NS). Additionally, SHAM mice had accurate spatial memory of the escape hole location: they quickly approached the escape hole location during a probe test (where the escape box was not available) 1 hr after the final training trial on day 3 (mean latency: $11.67 \pm 2.90$ s, significantly faster by $p < 0.05$ than mean latency to enter escape hole on day 3 training trials, $19.33 \pm 2.17$ s) (Fig. A.2A).

As we expected, IRR mice exhibited significantly slower learning (escape latencies) on the Barnes maze compared to SHAM mice that learned the location of an escape hole relatively rapidly (RMANOVA, main effect of IRR, $F_{(1,13)} = 2.99, \text{NS}$; main effect of day, $F_{(2,12)} = 15.18, p < 0.001$; day x IRR interaction, $F_{(2,12)} = 6.06, p < 0.02$) and overall did not show significant improvement in performance across training days (RMANOVA, main effect of day $F_{(2,10)} = 2.40, \text{NS}$; Fig. 2A). Like SHAM mice and unlike IRR mice, IRR-RUN mice showed a significant decrease in latency across training days (RMANOVA, main effect of day $F_{(2,6)} = 5.32, p < 0.05$; Fig. 2A). However, IRR-RUN mice
were overall an intermediate group, such that they were not significantly different in learning from the SHAM or IRR conditions when compared directly (RMANOVA, main effect of day, $F_{(2,19)} = 19.01, p < 0.0001$; day x condition interaction, Wilks' Lambda approx. $F_{(4,38)} = 3.24, p < 0.05$; followed by canonical-correlation analysis and Tukey-Kramer HSD post-hoc tests: SHAM vs. IRR, $p < 0.01$, SHAM vs. IRR-RUN, NS, IRR vs. IRR-RUN, NS).

Despite differences in the learning curves, there were no significant differences among SHAM, IRR, and IRR-RUN mice in their initial approach latency to the target hole on the 1 hr memory probe (one-way ANOVA, $F_{(2,20)} = 3.28, NS$). The accurate memory retention of IRR mice is further evident by their spending significantly more time in the target quadrant than other quadrants during the probe trial (one-way ANOVA, $F_{(3,44)} = 8.93, p < 0.0001$) (Fig. A.2B). IRR-RUN mice also spent more time in the target quadrant compared to all other quadrants during the 1 hr probe ($F_{(3,20)} = 7.07, p < 0.01$) (Fig. A.2B).
Figure A.2. Running reduced WBI-induced learning and memory deficits.

(A) Mean latency to target hole location on BM1 training days 1, 2, and 3 and the 1 hr probe. (B) Probe test performance 1 hr after BM1 training. The first bar in each probe test cluster represents the target quadrant, followed by adjacent right, opposite, and adjacent left. A target quadrant bias was evident if the percent time spent in the target quadrant was significantly greater (*p < 0.05) than all other quadrants. (C) Mean latency to new target hole location on BM2 training days 1, 2, and 3. (D) Probe test performance 1 hr after BM2 training. *, significantly different from all other quadrants at p < 0.05. (E) All mice showed a significant increase in mean escape latency from the last training trial where spatial cues were visible (cues present) to the trial where spatial cues were occluded by a curtain (cues removed). *, significantly different from “cues present” at p < 0.05.

Running recovered the deleterious effect of WBI on spatial retention in NSG mice

One month after initial Barnes maze training, all irradiated mice were able to learn a new escape hole location, showing decreasing escape latencies across days (RMANOVA, main effect of day, $F_{(2,17)} = 5.97, p < 0.02$; Fig. 2C). Running had no effect on the rate of reversal learning (day x running interaction, $F_{(2,17)} = 0.68, NS$). Probe
testing 1 hr following training revealed that IRR mice had poor memory retention for the new target quadrant (one-way ANOVA, $F_{(3,36)} = 5.01, p < 0.01$ followed by Tukey-Kramer HSD post-hoc test: target vs. left adjacent quadrant, NS; Fig. 2D). In contrast, IRR-RUN mice displayed accurate memory retention for the new escape hole location on the 1 hr probe ($F_{(3,16)} = 17.55, p < 0.0001$, followed by Tukey-Kramer HSD post-hoc test: target vs. all other quadrants, $p < 0.001$).

In order to determine if all mice were using hippocampal-dependent relational cues to locate the escape hole, mice underwent a trial wherein all extramaze cues were obscured by a circular curtain. If mice were using extramaze cues for navigation, performance should be greatly disrupted by obscuring these cues. We found that latency to enter the escape hole increased similarly for IRR and IRR-RUN mice compared to their last training trial of BM2 (RMANOVA, main effect of running, $F_{(1,18)} = 1.07$, NS; main effect of curtain, $F_{(1,18)} = 11.56, p < 0.01$; curtain x running interaction, $F_{(1,18)} = 0.18$, NS; Fig. 2C), indicating that mice were using hippocampal-dependent place processing for navigation as opposed to a stimulus-response strategy that would not require hippocampal processing (Morris et al., 1982).

**WBI reduced the number of proliferating cells, while running increased the number of newborn neurons in the dentate gyrus**

To determine whether WBI and running had long-lasting effects on cell proliferation, the number of PCNA+ proliferating cells in the dentate gyrus of the hippocampus was assessed two months following brain irradiation. While WBI significantly reduced the number of proliferating cells by 70% (one-way ANOVA, $F_{(2,18)} = 28.60, p < 0.0001$ followed by Tukey-Kramer HSD post-hoc test: SHAM vs. IRR, $p < 0.0001$; Fig. 3A), there was no lasting effect of running on cell proliferation (IRR vs.
To determine if WBI and running had a long-lasting influence on the survival of young neurons in the dentate gyrus, we assessed the number of cells labeled by DCX two months after WBI. We found that the number of DCX+ new neurons was decreased by 76% two months following WBI (one-way ANOVA, $F_{(2,19)} = 42.47, p < 0.0001$ followed by Tukey-Kramer HSD post-hoc test: SHAM vs. IRR, $p < 0.0001$; Fig. 3B & 3C) and running significantly increased the number of young neurons in the dentate gyrus of WBI mice by 74% (IRR vs. IRR-RUN, $p < 0.02$; Fig. 3B & 3C). Despite this large increase in new neurons, IRR-RUN mice still had 58% fewer DCX+ cells compared to the non-irradiated SHAM group (SHAM vs. IRR-RUN, $p < 0.0001$).
Running increased the survival of young neurons, but not cell proliferation, in the hippocampus two months after WBI. (A) Mean number of PCNA+ cells in the dorsal dentate gyrus. *, significantly different from SHAM at $P < 0.001$. (B) Mean number of DCX+ cells in the dorsal dentate gyrus. *, significantly different from SHAM at $P < 0.0001$. #, significantly different from IRR at $P < 0.02$. (C) Photomicrographs of DCX+ cells in the dentate gyrus from a representative SHAM, IRR, and IRR-RUN mouse.

Running increased growth factors in the hippocampus of irradiated NSG mice

In order to determine the factors that might contribute to the increased number of new neurons in the IRR-RUN mice compared to sedentary IRR mice, we examined hippocampal levels of several growth factors known to be important for the survival of new neurons. Voluntary running partially recovered hippocampal BDNF (one-way ANOVA, $F_{(2,20)} = 7.95, p < 0.01$ followed by Tukey HSD post hoc tests: SHAM vs. IRR, $p <$
Hippocampal IGF-1 was increased following WBI and was further increased by running ($F_{(2,20)} = 26.90, p < 0.0001$ followed by Tukey HSD post hoc tests: SHAM vs. IRR, $p < 0.001$, SHAM vs. IRR-RUN, $p < 0.001$, IRR vs. IRR-RUN, $p < 0.01$) to levels closer to SHAM mice (Fig. A.4A).

Figure A.4. Irradiation and voluntary running alter protein expression in the hippocampus two months after WBI. (A) Mean pg of growth factors per g hippocampal tissue for BDNF, VEGF, and IGF-1. *, significantly different from SHAM at $p < 0.05$. #, significantly different from IRR at $p < 0.05$. (B) Mean pg of inflammatory cytokine per g tissue for TNF-α, IFN-γ, IL-1β, IL-5, and IL-10. *, significantly different from SHAM at $p < 0.05$. #, significantly different from IRR at $p < 0.05$. (C) Mean pg of microglia marker, Iba1, per g hippocampal tissue. *, significantly different from SHAM at $P < 0.05$. ^, significantly different from IRR by Student’s t-test, $p < 0.05$. 

0.05, SHAM vs. IRR-RUN, NS, IRR vs. IRR-RUN, NS) and VEGF ($F_{(2,20)} = 17.32, p < 0.0001$ followed by Tukey HSD tests: SHAM vs. IRR, $p < 0.001$, SHAM vs. IRR-RUN, $p < 0.05$, IRR vs. IRR-RUN, $p < 0.01$) to levels closer to SHAM mice (Fig. A.4A).
Running regulated expression of inflammatory markers in the hippocampus

Because it is well known that brain irradiation increases neuroinflammatory processes that might interfere or enhance hippocampal neurogenesis, we examined hippocampal levels of a number of key pro- and anti-inflammatory cytokines. Running mitigated the WBI-induced increase in hippocampal expression of pro-inflammatory cytokines TNF-α (one-way ANOVA, $F_{(2,20)} = 39.49, p < 0.0001$ followed by Tukey HSD post hoc tests: SHAM vs. IRR, $p < 0.0001$, SHAM vs. IRR-RUN, NS, IRR vs. IRR-RUN, $p < 0.0001$), IFN-γ ($F_{(2,20)} = 43.26, p < 0.0001$ followed by Tukey HSD post hoc tests: SHAM vs. IRR, $p < 0.0001$, SHAM vs. IRR-RUN, NS, IRR vs. IRR-RUN, $p < 0.0001$), and IL-1β ($F_{(2,20)} = 7.93, p < 0.01$ followed by Tukey HSD post hoc tests: SHAM vs. IRR, $p < 0.01$, SHAM vs. IRR-RUN, NS, IRR vs. IRR-RUN, NS) (Fig. A.4B).

WBI also increased the expression of microglia marker Iba1 ($F_{(2,20)} = 10.23, p < 0.001$ followed by Tukey HSD post hoc tests: SHAM vs. IRR, $P < 0.001$, SHAM vs. IRR-RUN, $p < 0.02$) (Fig. A.4C). Although the effect of running was not significant when all three groups were compared in an ANOVA, there was a significant decrease in Iba1 when IRR and IRR-RUN groups were compared directly (Student’s t-test, $p < 0.05$).

A different expression pattern was observed for anti-inflammatory cytokines IL-5 and IL-10 (Fig. A.3B). While WBI did not induce any changes, running resulted in an increase in IL-5 (one-way ANOVA, $F_{(2,20)} = 19.38, p < 0.0001$ followed by Tukey HSD post hoc tests: SHAM vs. IRR, NS, SHAM vs. IRR-RUN, NS, IRR vs. IRR-RUN, $p < 0.0001$) and IL-10 ($F_{(2,20)} = 31.77, p < 0.0001$ followed by Tukey HSD post hoc tests: SHAM vs. IRR, NS, SHAM vs. IRR-RUN, $p < 0.0001$, IRR vs. IRR-RUN, $p < 0.0001$) (Fig. A.4B).
Discussion

Using NSG mice, which have constitutive deficits in innate and adaptive immunity, we investigated whether daily voluntary running would be an effective treatment for the neurocognitive deficits seen following whole-brain irradiation. We found that NSG mice, which have compound immunodeficiencies that include the absence of mature T cells, B cells, and NK cells (Shultz et al., 2005), were able to learn and remember an escape location on the Barnes maze. Furthermore, we found the lack of these immune cells did not prevent voluntary running from providing neuroprotective effects on hippocampal neurogenesis and memory following WBI. Even though NSG mice are highly immunodeficient, their basal neurocognitive function and hippocampal neurogenesis and their neural and cognitive response to exercise are similar to those of immunocompetent C57BL/6 mice that are subjected to WBI and daily voluntary running (Wong-Goodrich et al., 2010). Taken together, these data suggest that the maintenance of hippocampal neurogenesis and hippocampal-dependent spatial memory are T cell, B cell, and NK cell independent, as is the enhancement of hippocampal function by voluntary running. We conclude that aerobic exercise is an effective strategy for protecting against the decline in hippocampal neurogenesis and spatial memory following whole-brain irradiation, even in immunocompromised individuals.

NSG mice do seem somewhat more vulnerable to the learning deficits induced by WBI compared to mice with intact immune systems. In our previous study, irradiated C57BL/6 mice reached performance equivalent to non-irradiated mice by the third day of training on the Barnes maze within the first two weeks post-WBI (Wong-Goodrich et al., 2010). In our first timepoint, irradiated NSG mice showed slower initial learning such that even after three days of training, their performance was poorer than non-irradiated mice. However, both C57BL/6 and NSG mice similarly displayed deficits in
memory following reversal learning, which is perhaps a more stringent measure of hippocampal flexibility as it requires animals to forget a previous escape hole and remember a new spatial location on the same maze. Changes in levels of neurogenesis in response to WBI and running are also comparable between C57BL/6 and NSG mice. While there were slight differences in experimental design between the studies, irradiation induced a 70% reduction in the survival of BrdU+ cells in the hippocampus of C57BL/6 mice four months post-WBI and a 76% reduction in DCX+ young neurons in NSG mice two months post-WBI. Daily voluntary running following WBI was able to enhance the amount of BrdU+ cell survival by 87% in C57BL/6 mice compared to a 74% increase in DCX+ young neurons in NSG mice. Overall, the effects of immunodeficiency on hippocampal neurogenesis and spatial memory appear to be small in comparison to the severe deficits induced by WBI.

Also consistent with our previous findings in C57BL/6 mice, daily voluntary running partially recovers hippocampus neurogenesis, BDNF, and VEGF, and prevented the progressive WBI-induced deficits in hippocampal-dependent memory in NSG mice. Running also produces an elevation of hippocampal IGF-1 in both irradiated C57BL/6 and NSG mice. IGF-1, BDNF and VEGF have been described as mediators of exercise-induced neurogenesis and improvements in cognition (Trejo et al., 2001, Fabel et al., 2003, Vaynman et al., 2004, Ding et al., 2006, Cotman et al., 2007, Trejo et al., 2008). It is important to note that these NSG mice are capable of upregulating growth factors despite their severe immunodeficiencies.

While our data show rather small effects of immunodeficiency on neurocognitive function and response to exercise, prior research examining SCID mice (lacking T and B cells), nude mice (lacking T but not B cells), transgenic T\textsubscript{OV}A mice (with reduced CNS-specific T cells), and transgenic T\textsubscript{MBP} mice (with an excess of CNS-specific T cells)
indicate an important role for CNS-specific T cells in spatial learning and memory, environmental enrichment-induced increases in neurogenesis, and BDNF upregulation in the dentate gyrus (Ziv et al., 2006). There are several reasons why our data may differ from this prior report: 1) Morris water maze tasks have been shown to be more stressful for mice than the Barnes maze task. Water maze training induces greater plasma corticosterone than Barnes maze training, and perhaps more importantly, spatial learning is inversely correlated with corticosterone levels after water maze but not Barnes maze training (Harrison et al., 2009). As postulated by Ziv and colleagues (2006), T cell deficiency can exacerbate stress-related dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, which in turn, can impair both hippocampal neurogenesis and spatial memory. T cells are likely important for learning and neurogenesis under stressful conditions; however, our data support the view that a deficiency in T cells, even when compounded with other immunodeficiencies and irradiation, is not sufficient to block hippocampal-dependent spatial cognition and upregulation of neurogenesis from enriching stimuli like voluntary running. 2) It is also possible that the genetic background of the various strains of T cell-deficient mice tested by Ziv and colleagues (2006) contributed to the poor memory of Balb/C-T<sub>OVA</sub> mice on the water maze and the inability of Balb/C-SCID mice to upregulate neurogenesis in response to environmental enrichment. While the experimenters found positive results in Balb/C-wildtype mice compared to their transgenic counterparts, Balb/C mice are not generally recommended as background strain if the Morris water maze will be used to evaluate them due to these mice being strongly influenced by stressful conditions (Van Dam et al., 2006). 3) It is also possible that environmental enrichment is not a sufficiently potent stimulus to increase neurogenesis in T cell deficient mice. In fact, there is evidence that environmental enrichment and voluntary running act on the hippocampus via
dissociable pathways, with the later inducing a greater variety of neuroplastic changes than the former (Olson et al., 2006). Alternatively, it is possible that T cell deficient mice are able to display enrichment- or running-induced neuroprotection after an assault, such as WBI, but lack the capacity to enhance hippocampal function at a baseline.

The interaction of CNS-specific T cells with microglia had been hypothesized as a mechanism that regulates the expression of hippocampal growth factors and, therefore, neurogenesis (Ziv et al., 2006). However, there is more recent evidence that T cells and microglia do not interact in neurogenic niches. For instance, very few lymphocytes are detected locally in the rat SVZ following stroke, while there is a large increase in the number of microglia in the SVZ that express IGF-1 and class-II major histocompatibility complex (MHC-II), a microglia phenotype associated with neuroprotection (Ekdahl et al., 2009). The lack of lymphocytes in the SVZ is in contrast to the peri-infarct striatal area where lymphocytes are readily found. While voluntary wheel running increases hippocampal neurogenesis and microglia proliferation, neither MHC-II+ microglia nor T cells are detected in the local hippocampal microenvironment in either running or sedentary C57BL/6 mice (Olah et al., 2009). Therefore, it is unlikely that T cells play a direct role in the neuroprotective function of microglia following a neurological assault in the SVZ or SGZ.

Theories about the role of the immune system in neurogenesis are no longer focused on T cells but have expanded to consider the potential role of microglia and their ability to alter the profile of cytokines and growth factors in the hippocampus (Kohman and Rhodes, 2013). While NSG mice have a deficiency in cytokine signaling, irradiation and exercise are able to induce changes in the expression of cytokines. This finding could be explained by the fact that NSG mice lack the IL-2 receptor gamma gain, a receptor subunit common to only the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (Shultz
et al., 2005). We observed that running mitigates a WBI-induced increase in pro-inflammatory cytokines TNF-α, IFN-γ, and IL-1β and also leads to a potentially compensatory increase in anti-inflammatory cytokines IL-5 and IL-10 – none of which are directly affected by the NSG mutations. Brain cells that produce cytokines – such as endothelial cells, astrocytes, or microglia – could be responsible for regulating the production of different cytokines via compensatory mechanisms that are independent of T, B, and NK cells or gamma chain function.

As expected, we found that NSG mice exhibit an irradiation-induced increase in the amount of microglia-specific Iba1 protein in the hippocampus. This increase in Iba1 occurs as neuronal survival decreases following irradiation. Prior work has shown that the number of microglia in the dentate gyrus is inversely correlated with levels of adult hippocampal neurogenesis (Gebara et al., 2013), which is likely due to the role of microglia in the phagocytosis of dying cells in the region (Sierra et al., 2010). While voluntary running only results in a slight decrease in Iba1 in irradiated NSG mice, the anti-inflammatory cytokine profile induced by running suggests that it might be a change in the state of microglia activation, rather than the number of cells, that is responsible for the neuroprotective effects of exercise. This idea is consistent with the current understanding of microglia's modulatory role in neurogenesis, in which microglia can express either an inflammatory phenotype that suppresses cell proliferation, neuronal survival, and functional integration or a protective phenotype that enhances these aspects of adult neurogenesis (Kohman and Rhodes, 2013). For instance, a neuroprotective state of microglia is necessary for activating hippocampal neural precursor cells in response to voluntary wheel running (Vukovic et al., 2012). Differential ability to produce changes in microglia phenotype could provide another explanation for differences in NSG mice compared to other mouse models of T cell deficiency. Overall,
our findings demonstrate that hippocampal responsiveness to voluntary exercise is not fully dependent on T cells, B cells, NK cells, or IL-2 receptor signaling, such that other mechanisms, such as the modulation of cytokines secreted from microglia, must be responsible for exercise-induced enhancements of hippocampal neurogenesis and neurocognition.

Our findings that the immunodeficient hippocampal microenvironment is still very responsive to the beneficial effects of running suggests that exercise may be a promising avenue of treatment for cancer patients who have been immunocompromised by chemotherapy or irradiation. In fact, radiation therapy and chemotherapy are often given in combination as an effective treatment for malignant tumors and as a prophylaxis for brain metastases (Lee et al., 2012, Nabors et al., 2013). These cancer treatments are also associated with neurocognitive deficits that impair quality of life in cancer patients and are predictors of survival independent of tumor progression (Meyers et al., 2004, Li et al., 2008, Monje, 2008, Douw et al., 2009, Marko and Weil, 2010). Our findings strongly suggest that exercise may aid recovery or provide neuroprotection against the progressive loss of memory function, even in immunocompromised patients.
References


safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. Journal of Clinical Oncology 17:2639-2639.


in the hindlimb of rats selectively bred for innate low running capacity. Clinical Science 124.


Mitsukawa K, Mombereau C, Lötscher E, Uzunov DP, van der Putten H, Flor PJ, Cryan JF (2006) Metabotropic glutamate receptor subtype 7 ablation causes dysregulation of the HPA axis and increases hippocampal BDNF protein levels:


endurance running is protective against high-fat diet-induced insulin resistance. American Journal of Physiology-Endocrinology and Metabolism 293:E31-E41.


132


Biography

Christina Maria Tognoni was born on June 16, 1987 in Winchester, Massachusetts. In May of 2005, she graduated from Northview High School in Johns Creek, Georgia. She double-majored in Neuroscience and Spanish at Wellesley College in the town of Wellesley, Massachusetts, where she received a Bachelor of Arts and graduated cum laude with departmental honors in Neuroscience in June of 2009. She received a Masters of Arts in Psychology from Duke University in Durham, North Carolina in September of 2012 and became a Doctor of Philosophy in May of 2014, graduating from the Systems & Integrative Neuroscience Program in the department of Psychology & Neuroscience at Duke University.

Publications

Tognoni CM, Flores CT, Wulsin AC, Babb EA, Jones LW, Williams CL. Voluntary running can protect against whole-brain irradiation-induced neurocognitive deficits under severe immunodeficiency. [Submitted]


Honors & Awards

- "Best in Show," Duke Consortium of Neuroscience Graduate Programs (2012)
- Awarded Fellowship, NSF-GRFP (2011)
- Honorable mention, NSF-GRFP (2010)
- Endocrine Society Trainee Day Award (2009)
- Sigma Xi, Scientific Research Society (2009)
- Sigma Delta Pi, Hispanic Honor Society (2009)
- The Jorge Guillén Prize in Spanish Studies (2009)
- Organic Chemistry Award, Wellesley College (2008)
- HHMI award, Wellesley Science Center Summer Research Program (2008)

Professional Memberships

- International Stress & Behavior Society (ISBS)
- Society for Neuroscience (SfN)
- Endocrine Society