Neurogenesis is Altered Between and Across Brain Regions After Neurodegeneration and Injury

Alice Perez
The Graduate Center, City University of New York
NEUROGENESIS IS ALTERED BETWEEN AND ACROSS BRAIN REGIONS AFTER NEURODEGENERATION AND INJURY

by

ALICE PEREZ, M.A.

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2019
Neurogenesis is altered between and across brain regions after neurodegeneration and injury

by

Alice Perez

This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

__________________________
Date
Carolyn Pytte
Chair of Examining Committee

__________________________
Date
Richard Bodnar
Executive Officer

Supervisory Committee:
Dr. Nancy Foldi
Dr. Jeff Beeler

Readers:
Dr. Desiree Byrd
Dr. Joshua Jessel

CITY UNIVERSITY OF NEW YORK
ABSTRACT

Neurogenesis is altered between and across brain regions after neurodegeneration and injury

by

Alice Perez

Advisor: Dr. Carolyn L. Pytte

Adult neurogenesis is the addition of new neurons in the adult brain. In mammals, neurogenesis is largely restricted to the hippocampus and olfactory bulb, which underlie learning, memory, and olfaction. In songbirds, new neurons are incorporated into telencephalic regions that subserve the production, perception, and maintenance of song. Studies of the regulation of neurogenesis have exclusively focused on a single brain region and within a single hemisphere. Interestingly, it has yet to be investigated whether there are correlations between numbers of new neurons across regions and hemispheres within the same animal. Here we examined these differences in a rat model of a neurodegenerative disease and in songbirds with peripheral nerve damage. We found that neurogenesis is regulated across brain regions and within hemispheres of both animal models. An understanding of how neurogenesis is impacted across and within healthy and diseased brains may lead to strategies that consider interconnected effects in a whole brain system to help promote neurogenesis, which may ultimately mitigate cognitive impairment.
# TABLE OF CONTENTS

Abstract......................................................................................................................... iv

Chapter 1: Background .................................................................................................. 1

Chapter 2: New Neurons are Correlated with Timing Behavior ................................. 4

Huntington’s disease .................................................................................................... 4
Adult neurogenesis and its implications in Huntington’s disease ............................... 5
Basal ganglia pathology in Huntington’s disease ......................................................... 7
Hippocampal pathology in Huntington’s disease ......................................................... 8
Time perception ............................................................................................................. 9
  Timing deficits in Huntington’s disease .................................................................... 9
  Corticostriatal circuitry underlies timing behavior ................................................. 12
  The hippocampus underlies timing behavior ......................................................... 13
  Lateralization ............................................................................................................. 16

Aims ............................................................................................................................... 17

Materials and Methods ............................................................................................. 18
  Animals ....................................................................................................................... 18
  Operant chambers .................................................................................................... 19
  Behavioral training .................................................................................................. 19
  Behavioral data analysis ......................................................................................... 23
  Immunohistochemistry .......................................................................................... 24
  Microscope analysis .............................................................................................. 25
  Lateralization index ............................................................................................... 26
  Statistical analysis ................................................................................................ 26
    Cohort effects ....................................................................................................... 27
    Timing behavior ................................................................................................... 27
    Age effect ............................................................................................................. 27
    New neurons ......................................................................................................... 27

Timing behavior .......................................................................................................... 27

New neurons in the hippocampus and SVZ ................................................................. 27
  Comparing new neurons between genotypes .......................................................... 27
  Comparing new neurons between hemispheres ....................................................... 28
  Comparing new neurons across subregions ............................................................ 28

Timing behavior and new neurons in the hippocampus and SVZ ............................ 28
  SGZ, hilus, SGZ+hilus, and SVZ ............................................................................. 28
  Lateralization and timing behavior ...................................................................... 28
Results ......................................................................................................................... 29
Cohort effects ............................................................................................................... 29
Timing behavior ......................................................................................................... 29
Age effect .................................................................................................................. 29
New neurons .............................................................................................................. 30
Timing behavior ......................................................................................................... 30
New neurons in the hippocampus ............................................................................. 31
Comparing new neurons between genotypes ......................................................... 31
Comparing new neurons between hemispheres ..................................................... 31
Comparing new neurons across subregions ........................................................... 32
Timing behavior and new neurons in the hippocampus .......................................... 32
SGZ ............................................................................................................................... 32
Hilus ............................................................................................................................. 32
SGZ+hilus ................................................................................................................... 33
Lateralization ............................................................................................................. 34
New Neurons in the proliferative zones: SGZ and SVZ ......................................... 34
New Neurons in the Subventricular Zone (SVZ) .................................................... 34
Comparing new neurons between genotypes ......................................................... 34
Comparing new neurons between hemispheres ..................................................... 35
Timing Behavior and New Neurons in the SVZ ...................................................... 35
Discussion ................................................................................................................ 36
Time cells in the hippocampus ............................................................................... 37
New neurons are detrimental to timing performance in WT rats ......................... 38
Hippocampus ............................................................................................................ 38
SVZ ............................................................................................................................... 40
New neurons are beneficial to timing performance in diseased brains ............... 41
Hippocampus ............................................................................................................ 41
Left-sided lateralization of new neurons improves behavior ............................. 42
Limitations to the current study ............................................................................. 42
Validity of doublecortin as a marker of adult neurogenesis ................................ 42
Age of tgHD rats at the time of sacrifice ............................................................... 43
Chapter 3: Altered Sensory Feedback Impacts New Neurons in a Region and Hemisphere-Specific Manner

Materials and Methods

Results

Discussion

Chapter 4: General Discussion
The role of neurogenesis in neurodegenerative disease ................................................................. 105
Sex differences in brain lateralization ............................................................................................... 107
Conclusion ....................................................................................................................................... 108

References ...................................................................................................................................... 109
List of Figures

Figure 1. The hippocampus ................................................................. 44
Figure 2. Hippocampal subregions, subventricular zone and striatum .... 46
Figure 3. Correlation of new neurons across SGZ hemispheres........... 47
Figure 4. New neurons across the SGZ and hilus ............................... 47
Figure 5. Relationship between timing behavior and new neurons in the hilus ............................................................. 48
Figure 6. Relationship between timing behavior and new neurons in the SGZ+hilus ................................................................. 49
Figure 7. Timing behavior and lateralization of new neurons ............... 50
Figure 8. New neurons across the SGZ and SVZ ............................... 51
Figure 9. Correlation of new neurons across SVZ hemispheres ......... 51
Figure 10. Relationship between timing behavior and new neurons in the SVZ ................................................................. 52
Figure 11. Diagram of the song system .............................................. 82
Figure 12. Doublecortin-positive neuron in HVC labeled with DAB .... 82
Figure 13. BrdU/Hu stained neuron in HVC ........................................ 83
Figure 14. Song accuracy scores across treatment groups ................. 83
Figure 15. Song mean percentage similarity scores across treatment groups ................................................................. 84
Figure 16. 27 – 30 day old neurons in HVC across treatment groups ... 85
Figure 17. Lateralization of 27 – 30 day old neurons in HVC across treatment groups ................................................................. 85
Figure 18. 1 – 2 week old neurons in HVC across treatment groups .... 86
Figure 19. 27 – 30 day old neurons in Area X across treatment groups ... 86
Figure 20. Combined hemispheres of 27 – 30 day old neurons in Area X across treatment groups ................................................................. 87
Figure 21. Lateralization of 27 – 30 day old neurons in Area X across treatment groups ... 87
Figure 22. 1 – 2 week old neurons in Area X across treatment groups .... 88
Figure 23. Lateralization of 1 – 2 week old neurons in Area X across treatment groups .... 88
Figure 24. 27 – 30 day old neurons in NCM across treatment groups .... 89
Figure 25. Lateralization of 27 – 30 day old neurons in NCM across treatment groups .... 89
Figure 26. 1 – 2 week old neurons in NCM across treatment groups ........ 90
Figure 27. Lateralization of 1 – 2 week old neurons in NCM across treatment groups .... 90
Figure 28. Correlation of 27 – 30 day old neurons across hemispheres in HVC ................................................................. 92
Figure 29. Correlation of 27 – 30 day old neurons across hemispheres in Area X ................................................................. 94
Figure 30. Correlation of 27 – 30 day old neurons across hemispheres in NCM ................................................................. 95
Figure 31. Correlation of 1 – 2 week old neurons across hemispheres in HVC ................................................................. 97
Figure 32. Correlation of 1 – 2 week old neurons across hemispheres in Area X ................................................................. 99
Figure 33. Correlation of 1 – 2 week old neurons across hemispheres in NCM ................................................................. 100
List of Tables

Table 1. Comparing new neurons between genotypes.........................................................31
Table 2. Correlations in 27 – 30 day old neurons between brain regions within
hemispheres ..................................................................................................................73
Table 3. Correlations in 1 – 2 week old neurons between brain regions within
hemispheres..................................................................................................................73
CHAPTER 1: BACKGROUND

The discovery of adult neurogenesis in mammals occurred over 50 years ago, shifting our understanding of how the central nervous system functions in health and disease (Alvarez-Buylla and Lim, 2004). Adult neurogenesis is the postnatal formation of new neurons in the brain. Significant progress has been made in understanding neurogenesis, ranging from proliferation to the integration of newborn neurons in brain regions that receive them. The process of adult neurogenesis is tightly regulated and can be influenced not only by genetic factors, but also physiological, pathological, and behavioral factors (Gould et al., 1999; Deng et al., 2010; Aimone et al., 2014). The multitude of ways in which neurogenesis is regulated raises the hope that therapeutic interventions may increase new neuron production and survival, thereby potentially compensating for neurons that have been lost through disease and brain injury. Furthermore, studies have illustrated the functional impact of new neurons under both healthy and damaged states (Alvarez-Buylla and Lim, 2004). In spite of such progress, differences in neurogenesis across and within brain regions have yet to be examined within an animal. Disease and injury are two predominant means by which neuronal loss may impact neurogenesis, new neuron recruitment, and new neuron survival. Interestingly, as in the healthy brain, model systems of disease and injury have thus far ignored potential interactions in neurogenesis in multiple regions of the brain. As a first step toward understanding whole-brain neurogenesis, I investigated whether neurodegeneration and altered sensory feedback via peripheral nerve injury impacted new neurons similarly across brain regions within the same hemisphere, and also in the same region between the two hemispheres, using a Huntington’s disease rat model and a songbird model. The examination of inter- and intra- hemispheric effects of new neurons after disease and injury provide an understanding of whether new neurons are regulated uniformly in
the brain and/or impacted similarly following brain damage. Ultimately, the goal is to inform appropriate hemisphere- or region-specific treatment options for the reversal of cognitive impairments that are associated with neuronal loss that occurs through disease and injury.

A comparative analysis of differences in new neuron numbers across brain regions may potentially inform treatment options in a disease-specific manner. For instance, newborn neurons added to hippocampal circuitry are important for learning and memory (Barnea and Nottebohm, 1994; Gould et al., 1999) of specific tasks (Shors et al., 2001; Shors et al., 2008; Dupret et al., 2008; Fan et al., 2007; Raber et al., 2004; Imayoshi et al., 2008; Farioli-Veccio et al., 2008; Zhang et al., 2008). However, the functional role of new neurons in other regions (e.g., subventricular zone, striatum, cortex, cerebellum, amygdala) has yet to be investigated (Alvarez-Buylla et al., 2002; Ernst et al., 2014; Gould et al., 1999; Carletti & Rossi, 2008; Bernier et al., 2002). In a disease that predominantly affects the hippocampus, such as Alzheimer’s disease, the vast majority of studies report that an increase in disease biomarkers, as well as greater cognitive deficits, coincides with a decrease in neurogenesis (Lazarov & Marr, 2010; Kuhn, 2007). Yet, in diseases in which the hippocampus is not the primary targeted region, it is unknown whether there is a decrease, increase, or absence of change, in numbers of new neurons, or which brain regions (if any) are affected. Given that different diseases have specific accompanying brain pathologies, the question of whether new neurons are affected similarly across brain regions after neuronal loss warrants understanding, as it can provide therapeutic approaches based on pathology and disease.

Functional inter-hemispheric differences of various brain regions have been explored in both diseased and non-diseased states (Geschwind, 1971; Damasio, 1971; Benson and Ardila, 1996; Karnath 2001, 2004). For instance, in humans, the left perisylvian region is specialized in
language processing (Geschwind, 1971; Damasio, 1992; Benson and Ardila, 1996), while the right perisylvian network is more specialized in spatial orienting (Karnath, 2001). In addition to visuospatial abilities, various right hemispheric structures underlie attention, musical abilities, and many aspects of emotion (Heilman & Van Den Abell, 1980; (Zatorre et al., 1994; Tervaniemi et al., 2000; Borod, Cicero, & Obler, 1998; Adolphs, Jansari, & Tranel, 2001).

During a disease process, such as Alzheimer’s disease (AD), left sided asymmetry of language processing is significantly reduced. Furthermore, while performing semantic and episodic tasks, patients with AD have been shown to recruit bilateral prefrontal areas rather than the left ventrolateral prefrontal cortex, which is recruited in healthy controls. In spite of these differences in functional lateralization, it is less clear whether there are also inter-hemispheric differences in neurogenesis across multiple brain regions. Tsoi et al. (2014) conducted the first study to examine the relationship between lateralization of neurogenesis and behavior. It was found that the degree of left side lateralization of new neuron incorporation in a region that underlies auditory memory in zebra finches was correlated with the quality of song. This finding suggests that neurogenesis may serve a different function, and be differentially regulated, across hemispheres. Whether differences in hemispheric lateralization of new neurons exist in other avian brain regions, or in brain regions of other taxa, has not yet been examined. An understanding of inter-hemispheric differences in neurogenesis would help in determining whether a specific hemisphere has a greater contribution to neuroplasticity or brain repair. Understanding the role that new neurons play in cognitive tasks from both left and right hemispheres could also enhance the specificity of stem cell transplant therapies that are currently being used for endogenous brain repair (Horie et al., 2015; Kalladka & Muir, 2014).
My dissertation is the first to demonstrate that region and hemisphere-specific differences in neurogenesis exist after neurodegeneration and altered sensory feedback via peripheral nerve injury. In a transgenic rat model of Huntington’s disease (HD), it was also found that the relationship between neurogenesis and a behavior commonly affected in HD (i.e., timing) differed within hemispheres and across regions that receive new neurons and underlie timing behavior. The impact of altered sensory feedback on new neurons was examined in a songbird model after song was altered via tracheosyringeal denervation. This injury resulted in differences in neurogenesis within hemispheres and across regions that underlie song behavior. The results from both studies provides insight into the plastic nature of the brain, and allows for an understanding of whether plasticity occurs on a global “whole-brain” level under these conditions. These findings provide further information on the continuous structural rearrangement of the brain and whether it occurs similarly within different hemispheres and across different regions. Understanding this process from a neuronal level may ultimately contribute to new treatment strategies for treatment of neurological and psychiatric disorders.

CHAPTER 2: NEW NEURONS ARE CORRELATED WITH TIMING BEHAVIOR

*Huntington’s disease*

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expansion of CAG repeats in the coding region of the huntingtin gene on chromosome 4, resulting in a protein with an abnormally long polyglutamine sequence (The Huntington’s Disease Collaborative Research Group, 1993). Mutated huntingtin binds to transcription factors and accumulates in neurons causing dysfunction and eventual cell death. It is a progressive and fatal disease characterized by cognitive, affective, and motor dysfunction. Symptoms usually
occur in midlife (35-60 years) and lead to death 15-20 years after their onset (Walker, 2007). The clinical diagnosis of HD relies on the manifestation of motor abnormalities, which includes atypical saccadic eye movements with increased latency and increased variability, slow and variable arm movements, slow asymmetric gait with increased force, and timing variability during precision grip (Rao, 2014). Variability in timing is one feature that is common to the motor impairments present in the disease. Motor impairments are evident in the form of hyperkinetic movements that occur in the early stages of HD, which may be a result of increased DA transmission (Garrett & Suarez de Silva, 1992), as well as hypokinetic movements that occur in the later stages of the disease that are possibly due to decreased DA transmission (Kish, 1987).

**Adult neurogenesis and its implications in Huntington’s disease**

In the mammalian brain, neurogenesis mainly occurs in two regions: the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. Neurons born in the SGZ migrate into the adjacent granular layer of the DG, where they become granular neurons, and seem to play a role in hippocampal-dependent learning and memory (Nilsson et al., 1999; Santarelli et al., 2003; Enwere et al., 2004; Kempermann et al., 2004). In the SVZ of rodents, there is evidence that neural stem cells give rise to neuroblasts, which migrate anteriorly into the olfactory bulb, where they mature into local interneurons (Altman, 1969; Lois and Alvarez-Buylla, 1994; Pencea et al., 2001), and contribute to correct processing of olfactory inputs. The adult human SVZ also contains neuroblasts but neurogenesis has not been detected in the human olfactory bulb. A bulk of experimental data has now also established that neural stem cells in the SVZ of humans and rodents also migrate into the
striatum and differentiate to mature neurons (Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2003; Ernst et al., 2014).

There are several factors governing the generation, migration, differentiation, integration and survival of new neurons in the SGZ and SVZ. Some of these factors include neurotransmitters (in particular, dopamine, serotonin, GABA, acetylcholine and glutamate; Hagg 2005; Lledo and Saghatelyan, 2005), making it possible that changes to neurotransmitters levels, as occurs in neurodegenerative diseases, would impact adult neurogenesis in the SGZ and SVZ with unknown functional consequences. Likewise, the discovery of adult neurogenesis has raised the hope that the SGZ and SVZ might have the capacity to generate neurons that can replace the ones lost through disease.

In disease states, such as Huntington’s disease, new neurons that originate in the SVZ are recruited to sites of damaged tissue, particularly the striatum (Batista et al., 2006; Kohl et al., 2010). This has been shown in HD animal models, in which neurons from the SVZ migrate ectopically to the degenerating area of the striatum – perhaps partially compensating for neuronal loss. Further, Curtis et al. (2003) described an increase in cell proliferation in the SVZ of human patients with HD. The increased number of new neurons correlated with the severity of the disease and the number of CAG repeats. New neurons were also found close to the caudate nucleus in patients with HD, which might further suggest potential migration of new neurons to the degenerating striatum.

Although an increased number of adult-formed neurons exist in the SVZ and striatal region of patients with HD, differences in new neuron numbers in the dentate gyrus could not be detected when comparing patients with HD with healthy controls (Low et al., 2011). However, several studies have reported reduced neuronal proliferation rates in the DG in both HD mouse
models (R6/1 and R6/2) and in a HD transgenic rat model with 51 CAG repeats (tgHD), which more closely reflects the human disease (Vlamings et al., 2012). Findings demonstrating increased numbers of new neurons in the SVZ and striatum, and decreased numbers of new neurons in the hippocampus of HD animal models suggest that brain regions may be differentially vulnerable to underlying neurodegenerative processes.

*Basal ganglia pathology in Huntington’s disease*

Neuropathological and imaging studies demonstrate that brain abnormalities in HD develop well before the onset of symptoms and eventually involve the entire brain, resulting in about 25% brain weight loss in advanced HD (Halliday et al., 1998, Sharp and Ross, 1996). The most widely studied structure affected in HD is the striatum. In this region, atrophy is accompanied by extensive neuronal loss and astrogliosis (an increased number of astrocytes in response to central nervous system disease), both of which become more severe as the disease progresses (Reiner, 2011). HD mouse models constructed by introducing CAG repeats also display a marked reduction in brain size that is accompanied by severe striatal atrophy. One contributing factor for this volume loss is the degeneration of fibers to and from the striatum. Medium spiny neurons (MSNs) are GABAergic neurons that make up 95% of the neurons in the striatum. In HD rodent models, MSNs display around 20% loss of soma area, decreases in their dendritic arborizations, reduced spine density, and overall loss in cell numbers (Klapstein et al., 2001). MSNs account for the reduction in striatal volume observed in HD animal models and human patients with HD (Schilling et al., 1999; Hodgson et al., 1999; Slow et al., 2003). Consequently, they have altered levels of dopamine, GABA, acetylcholine and glutamate (Andre, Cepeda, Levine, 2010). Other brain regions affected by neuronal loss and shrinkage in HD include the cerebral cortex and
hippocampus (Rosas et al., 2003). However, the neuropathology of these regions has been examined to a far lesser extent than the basal ganglia.

Hippocampal pathology in Huntington’s disease

In recent years the idea has emerged that cognitive decline in HD is more likely a reflection of defects in global brain circuitry rather than an exclusive dysfunction of the basal ganglia (Brito, 2016). In addition to loss of volume in the striatum, hippocampal volume is also reduced in presymptomatic and symptomatic HD individuals. Spargo (1993) revealed 35% cell loss in the hippocampus in patients with HD and showed the changes to be primarily targeted to area CA1 (Figure 1A). Strikingly, behavioral changes that rely on hippocampal function, such as spatial and episodic memories, are also altered in patients with HD. In moderately advanced patients with HD there is a simultaneous impairment of hippocampal-dependent and striatal-dependent spatial navigation (Majerova et al., 2012), supporting the idea that a more general neurodegenerative process that involves the hippocampus could contribute to HD cognitive impairment beyond the dysfunction of the striatum. Furthermore, cytoplasmic and nuclear huntingtin aggregates within the hippocampus have been described in patients with HD (Gil-Mohapel, et al., 2011). The spatial and episodic deficits, together with hippocampal long-term potentiation (LTP) reductions, have been described in different HD mouse models, with altered synaptic plasticity and aberrant dendritic spine density in the hippocampus as contributing mechanisms (Brito et al., 2014). The factors underlying hippocampal neuronal damage in HD remain unclear, although it has been proposed that decreases in dopamine signaling occur early on in the disease and may impact both neuronal loss and increased proliferation (Kippin et al., 2005).
Time perception

Organisms have developed multiple temporal processing systems, which vary in orders of magnitude, for adaptive reasons (Roberts & Church, 1978; Buhusi et al., 2005; Gallistel et al., 2004; Gribova et al., 2002). In humans, timing of behaviors is critical in everyday activities, particularly prominent in our sleep-wake cycle, speed of movement, motor control in walking, speech, musical activities, sports (Wearden & McShane, 1988; Buhusi & Meck, 2005; Grondin, 2010; Wittman & Wassenhove, 2009; Malapani, 2002), as well as in cognitive tasks such as in sequential planning and memory formation (Wittman & Wassenhove, 2016; Yin & Troger, 2011). Similar to other animals, we are able to use temporal information across various time scales, including sub-second range timing (milliseconds) and supra-second range timing (seconds to minutes) as well as longer durations. For example, we use circadian rhythms to operate over the range of the 24 hour light-dark cycle, and influence sleep and wakefulness (Buhusi & Meck, 2005); interval timing in the range of seconds to minutes is involved in foraging, reproductive behavior, decision-making, associative learning, and mental estimation of time (Gallistel, 1990; Malapani, 2002; Buhusi, 2009); likewise, millisecond timing is critical for motor control, speech generation and recognition, playing music, and countless voluntary motor behaviors such as in sports or dancing (Schirmer, 2004, Droit-Volet & Meck 2007; Eagleman, 2008; Wittmann & Paulus, 2008).

Timing deficits in Huntington’s disease

Increasing evidence suggests that cognitive deficits, and deficits in time perception, are present at least 15 years prior to an impairment in motor abilities in patients with HD (Paulsen, et al.,
Interestingly, timing abilities continue to decline with disease progression and may further compromise the motor performance of patients with HD, due to the involvement of timing mechanisms in the coordination of muscles. In addition, areas of cognitive impairment in HD that are interconnected with timing mechanisms include attention, language, memory, visuospatial functioning, initiation, and speed of processing. Given these interactions, it appears that disrupted temporal processing may also underlie the deficits found in these cognitive areas of patients with Huntington’s disease.

The relationship between cognition and timing behavior has been extensively investigated in both HD animal models and patients with HD. In the transgenic R6/2 mouse model of HD, Balci et al., (2009) reported disrupted temporal control and increased variability in a task assessing interval timing abilities. Likewise, HD carriers who approach (within < 12 years) the age at which they are predicted to develop HD (“HD-close”) perform worse in tests of interval timing than control individuals or patients whose predicted time of disease onset is more than 12 years away (“HD-far”) (Rao et al., 2014). Furthermore, fMRI found that those in the control group (i.e., non-carriers) showed activation in the caudate-putamen, thalamus, pre-supplementary motor area (pre-SMA) and cingulate cortex. Those in the HD-far group also showed activation in these regions, but with increased activation in the pre-SMA and caudate nucleus, possibly indicating compensatory activity explaining their relatively normal timing performance. Interestingly, patients in the HD-close group showed significantly decreased activation in all four regions.

A similar study examined whether timing performance is associated with proximity to diagnosis (Rao et al., 2014). A single interval production task was used, in which participants were asked to mark the end of a stimulus (i.e., tone) for which they had previously been trained
to detect the duration (i.e., 1.1s, 2.2s, or 3.3s). Participants with pre-manifest HD (i.e., movement disorder with expanded CAG allele >36, but no diagnosis of HD) and manifest HD (i.e., confirmed diagnosis of HD and mild functional limitations) had impaired precision timing compared with matched control subjects, and precision was related to proximity to diagnosis.

Timing deficits in HD have predominantly been investigated in the duration of milliseconds and seconds by tasks that require intact motor processing, such as keeping spontaneous rhythm and self-paced timing tasks (Hinton et al., 2007). Recent evidence also suggests impairments in time perception tasks irrespective of intact motor processing (Righi, 2016). For instance, it was found that individuals had time perception deficits for seconds and milliseconds in early to moderate stages of HD in a temporal bisection task, which required subjects to compare temporal stimuli to durations held in memory (Righi et al., 2016). Subjects overestimated the short stimuli durations and underestimated the long durations, displaying a “migration effect,” or a loss of memory of time. The findings from this study are consistent with timing deficits found in humans and animals with hippocampal damage (Meck et al., 1984, 1987; Olton et al., 1987, 1988; Buhusi et al., 2005; Balci et al., 2009; Vidalaki et al., 1999; Melgire et al., 2005). However, the direction of the error differs in patients and animal models of Huntington’s disease (which primarily affects the striatum) than in humans and animals with primary hippocampal damage. The difference in error directionality may suggest that the hippocampus and striatum play differential roles in timing systems, or the perception of these systems, and that there is an interaction between the regions during timing processing.

Despite the wealth of literature dedicated to examining the relationship between timing perception and cognitive functioning in HD (Righi et al., 2016; Beste et al., 2007; Agostino et al., 2016; Cope et al., 2014; Paulsen et al., 2004), there is still considerable debate about whether
poor timing performance in more severe HD is directly affected by cognitive impairment. Some researchers propose that motor symptoms exist in the absence of cognitive impairment in early HD (de Boo et al., 1997). Conversely, others argue that there exists cognitive decline in presymptomatic HD gene carriers (Lawrence et al., 1998; Hahn-Barma et al., 1998). Perhaps individual differences exist and are associated with the severity of the CAG repeat and specific genetic characteristics of HD. In summary, with regard to timing behavior, HD studies on animals and human patients have consistently demonstrated impairment in tasks that require intact time processing in presymptomatic and symptomatic subjects (Weardon & McShane, 1988; Balci et al., 2009; Rao et al., 2014; Hinton et al., 2007; Righi et al., 2016). Although this effect of the disease is quite clear, it still warrants further investigation in determining whether impaired time processing may in fact underlie impairment in other cognitive areas.

**Corticostriatal circuitry underlies timing behavior**

The striatum, thalamus, and prefrontal cortical areas are anatomically linked and functionally interconnected during timing tasks (Matell & Meck, 2004), indicating that cortico-striatal-thalamo circuits not only play a role in processing sensorimotor functions, emotions, and cognition, but also in interval timing. This connectivity was first demonstrated using human fMRI studies (Hinton et al., 1996).

fMRI activity in frontal-striatal circuits in the timing of seconds by humans was demonstrated using a peak-interval timing procedure (Rakitin et al., 1998), which requires participants to reproduce (e.g., by pressing a button) previously presented durations (Hinton & Meck, 2004). Activation of the frontal cortex, striatum, and thalamus was detected when participants timed and made a motor response (timing+motor task) and when they timed
internally without producing a motor response (timing task). A motor control task in which responses were not controlled by time did not show activation of the striatum, underscoring the importance of the striatum to the timing component of motor behavior.

A related study (Rao et al., 1997) used fMRI to image subjects while they performed a finger-tapping task while listening to an auditory cue and in the absence of this cue. Both tasks produced areas of activation in the left sensorimotor cortex and the right cerebellum, consistent with finger movements in the right hand (Rao et al., 1997). Interestingly, activation of the striatum (i.e., left caudal putamen), thalamus (i.e., left ventrolateral thalamus), and cortex (i.e., supplementary motor area) occurred only when the timing of the taps depended upon an internal representation of time, and activation of these regions did not occur when the participants were required to tap in time to the auditory cue. This suggests that these structures are critical to timing behavior. Similar effects have also been observed using positron emission tomography (PET) to map the basic patterns of activation in motor and sensory temporal tasks (Lejeune et al., 1997).

The hippocampus underlies timing behavior

The hippocampus is most commonly known for its involvement in spatial and episodic memory, with the latter including information about how long ago specific events occurred. This temporal element is crucial in distinguishing individual episodes, which is necessary given that most of our experiences have overlapping qualities (e.g. parking in the same parking lot on a daily basis). Electrophysiological studies have confirmed that individual hippocampal neurons in CA1 (Figure 1A) show timing signals (“time cells”; Pastalkova et al., 2008; MacDonald et al., 2011), and have demonstrated the importance of the hippocampus in timing abilities and behaviors.
The hippocampus is part of the medial temporal lobe, which includes a system of anatomically interconnected structures such as the perirhinal, parahippocampal, and entorhinal neocortical regions (Figure 1B). These structures operate on a representation of experience (episode) that includes information about not only which stimuli have been experienced, but also the time and order in which they were experienced (Meck, 2005).

Neuropsychological evidence related to the role of the medial temporal lobe in timing tasks was observed in the classic case of H.M., a male who underwent a bilateral medial temporal lobe resection that resulted in a severe memory loss following surgery. H.M. underwent formal testing for decades post-surgery in order to determine whether there were deficits in his cognitive function. One task required reproduction of durations between 1 and 300 s (5 min). H.M. demonstrated reasonably accurate timing for durations < 20 s, but systematic underestimation for durations > 20 s (Meck, 2005). Since then, a number of surgical procedures in rats and mice impacting hippocampal function (e.g., transection of the fimbria fornix, lesions of the medial septal area, resection of the temporal lobe, selective lesions of the dorsal hippocampus and destruction of the entire hippocampus) have been shown to result in substantial changes in interval timing accuracy (i.e., responding at the appropriate time) and precision (i.e., the time between start and stop times, or the stimulus control of behavior by time) (Meck, Church & Olton, 1984; Meck et al., 1987; Balci et al., 2009). It has been found that lesions to the hippocampus in rats and mice also result in a “leftward” shift (shorter duration) of timing judgments for intervals in the range of 10-40 s (Meck et al., 1984, 1987; Olton et al., 1987, 1988; Buhusi et al., 2005; Balci et al., 2009), and 2-8 s (Vidalaki et al., 1999; Melgire et al., 2005). Taken together, animal and human studies have shown that subjects with hippocampal damage
responded earlier than the scheduled time of reinforcement in a variety of timing tasks, suggesting that the hippocampus plays a role in temporal memory (Yin and Meck, 2014).

A currently debated question is whether the neural substrates involved in the temporal processing of durations in the millisecond range differ from those underlying processing durations in the seconds to minutes range (Yin & Troger, 2011; Lusk et al., 2016; Meck et al., 2013). A behavioral task was developed to investigate the ability of rats to remember the timing of events in the magnitude of several minutes (Jacobs et al., 2013). Surprisingly, it was found that hippocampal inactivation was beneficial in discriminating timescales separated by 60-90 second durations, but severely impaired performance requiring discrimination between 8 versus 12 min durations. This suggests that intact hippocampal processing is necessary when the timescale is greater than several minutes, but detrimental when the timescale is shorter (circa 1 min). Together with earlier work demonstrating that the hippocampus is necessary for accurate timing on the order of milliseconds, this suggests a potential window in which the hippocampus is not used (or impedes) perception of stimuli of ~60-90 second durations, but is necessary for shorter and longer time frames. Interestingly, the striatum is typically considered the brain region involved in timing ability in the range of seconds, thus suggesting that the hippocampus is not critical for timing at this scale (Dietrich et al., 1997; Kyd et al., 2008), and can competitively interact with other (e.g. striatal) timing systems (Meck, 2005).

It has also been more directly reported that a competitive interaction between hippocampal and striatal systems exists, such that damage to one of them can lead to facilitation in the other system (Poldrack and Packard, 2003). The factors that may contribute to this effect include direct anatomical projections from the hippocampus (i.e., entorhinal cortex) to the dorsal striatum (Matell, Meck, and Nicolelis, 2003; Sorensen & Witter, 1983), which may play a role in
interval timing, although it has yet to be demonstrated. In addition, studies on mouse and rat models have indicated that lesions to the ventral hippocampus can lead to increased dopaminergic transmission in the dorsal striatum (Lipska, Jaskiw, Chrapusta, Karoum, and Weinberge, 1992), which then produces deficits in the accuracy and precision of interval timing in the seconds-to-minutes range (Meck et al., 1984; Vidalaki et al., 1999; Meck 2005; Melgire 2005).

_Lateralization_

There are differences in both structure and function between left and right hemispheres of the hippocampus and striatum, suggesting hemispheric specialization in both brain regions (Gotts et al., 2013). Patients with unilateral medial temporal lobe lesions show increased PET activity in the right hippocampus that is positively correlated with navigation accuracy (Maguire et al., 1998; Spiers et al., 2011), while damage to the left hippocampus resulted in deficits of contextual memory (Spiers et al., 2011). Similarly, fMRI showed activity in the right, but not in the left hippocampus, that was consistent with conveying information regarding the distance to a goal location (Howard et al., 2014).

During some types of processing, it seems that hippocampal hemispheres work independently to carry out a function. In a similar vein, neuroimaging studies demonstrate that there is increased activation in the right striatum when participants are performing visuospatial and set-shifting tasks (Alivisatos & Petrides, 1997; Dong et al., 2000), whereas the left striatum is activated in response to language tasks (Caplan, et al., 1990; Desmond et al., 1998). Gotts et al., (2013) provided evidence that lateralization of function improves cognition and memory. Interestingly, lateralization of new neurons in the dentate gyrus of the hippocampus and the SVZ,
the region that supplies new neurons to the striatum, has yet to be investigated to determine whether the substrates may use new neurons differently, or to different degrees, in information processing between left and right hemispheres.

**Aims**

The aims of this study were to 1) compare numbers of new neurons in regions of the hippocampus (i.e., SGZ, hilus, and SGZ+hilus) and SVZ a between a transgenic Huntington’s disease rat model (tgHD) and control rats; 2) to compare numbers of new neurons within each genotype and within each region between hemispheres, and 3) to compare the relationship between new neurons in regions of the hippocampus (i.e., SGZ, hilus, SGZ+hilus) and SVZ with performance on a timing task. This study is the first to examine whether adult neurogenesis is altered across brain regions and between hemispheres in a tgHD rat model. This work has translational implications given that neurogenesis may play a role in cognitive functioning. For instance, a decrease in new neuron numbers in one or both brain regions would highlight the need for stimulation of the production of neurons to that region in order to reverse the cognitive effects characterized by the disease. On the contrary, a lack of difference in neurogenesis across brain regions or between hemispheres of tgHD and control rats may suggest that new neurons are independent of the cognitive deficits observed in the disease process.

This study is also the first to examine whether new neuron lateralization exists in the SVZ or hippocampal regions in both tgHD and control rats. Such a finding would suggest that asymmetrical differences are associated with either a disease process or normal brain functioning. These results would inform stem cell transplant therapies in determining whether a specific hemisphere has a greater contribution to brain repair versus the other hemisphere.
Lastly, an understanding of the relationship between new neurons and performance on a timing task would further our understanding of how new neurons contribute to behavior. These findings would ultimately allow us to understand whether the manipulation of new neurons may provide improved behavioral outcomes.

**Materials and Methods**

*Animals*

All experiments were conducted with approval of the Queens College Institutional Animal Care and Use Committee (IACUC) and followed National Institute of Health (NIH) animal care and use guidelines. Subjects were 14 adult male rats, 7 of which were genotyped (Laragen, Inc.) as homozygous dominant for the human huntingtin gene (tgHD) and 7 of which were wild-type (WT). Animals were divided into 3 cohorts based on litter: the first cohort included 5 rats (tgHD n = 3; WT n = 2); the second cohort included 4 rats (tgHD n = 2; WT n = 2); and the third cohort included 5 rats (tgHD n = 2; WT n = 3). The lifespan for both tgHD rats and WT rats is approximately 2 years (Savas et al., 2012). Phenotypically, tgHD rats display HD symptoms (e.g., gait and balance abnormalities, chorea-like movements) at around 6-8 months old (Casaca-Carreira et al., 2015). Animals were maintained in a climate-controlled room on a 12-hour daylight cycle, and housed individually in cages with ad libitum access to food pellets. Animals were partially deprived of water (1 hour of ad libitum access to water per day while in their home cages) one week prior to training in order to acclimate them to a deprivation schedule.
Operant chambers

We used 4 operant chambers with metal walls on two sides and clear plastic front and back walls and ceilings, and steel floor bars for training and testing procedures (30.5 cm wide × 24.1 cm long × 29.2 cm high, Lehigh Valley Electronics). Each chamber contained 1 house light, 1 cue light, a lever, and a dipper to deliver water reinforcement. The house light was located on the ceiling near the rear wall and was used as a timing signal. The cue light was located on the right wall and provided illumination throughout the session. The lever was located underneath the cue light on the right panel wall. The water dipper was centered on the right panel wall, adjacent to the lever. A fan and white noise generator were connected to each chamber and provided 10 kHz white noise at an average volume of 77 dB within boxes in order to attenuate room noise. Each chamber was connected to Med-Associates input and output cards in an adjacent room. The computer program “Med-PC IV” was used to control programming, send signals, and record events. Med-PC IV was run on a computer using a Microsoft Windows 98 operating system.

Behavioral training

Behavioral training consisted of four phases in the following order: dipper training, response training, fixed interval training and peak interval training. Training began between the ages of 2-3 months (young adulthood) and was completed by 3-6 months. Adulthood is reached at 6 months. Animals were randomly assigned into operant chambers at the start of dipper training. The time of day of experimental sessions was held constant for each subject. In order to maintain constant retention intervals between sessions, each training session began 24 hours after the onset of the previous training session. Daily training sessions lasted between 1-2 hours.
depending on individual performance and testing phase. A water deprivation schedule was maintained throughout the duration of all training phases.

**Dipper Training (1-2 sessions)**

Dipper training was conducted to train the rats that water was available in the chambers. Animals were placed in the operant chambers and received a drop of water via the dipper every 60 seconds (s) independent of responding, and additionally, for each individual press made on the lever. Sessions were terminated after one hour. The number of sessions conducted was dependent on the individual animal’s performance, measured as lever presses. After dipper training, animals were allowed one hour of free access to water in their home cage.

**Response Training (5 sessions)**

Dipper training was followed by response training, which was used to establish lever pressing. Animals were placed in operant chambers for one hour. During this time, animals could press the lever for access to water under a fixed ratio 1 schedule of reinforcement, for a maximum of 120 reinforcers before the session ended. To move onto the next training phase, animals had to successfully complete two consecutive sessions of 120 lever presses (there was a 2-hour maximum session length). For subjects that did not independently acquire the lever press response within 5 sessions, lever pressing was taught via shaping. Shaping is a stepwise process that involves manual depression of the lever by the researcher whenever the animal showed gradually progressive approximations towards lever pressing (e.g., moves toward the lever, puts its head near the lever, or puts its paw on top of the lever). The eventual goal of shaping is to train the animal to autonomously press the lever for water access. A fixed ratio 1 schedule is defined as a constant reinforcement delivery after every correct trial.
Fixed Interval Training (10-20 sessions)

Fixed interval (FI) training occurred after the completion of response training. During this phase, rats had to withhold responding for a certain amount of time before responding with a lever press. Animals were placed in the operant chambers for a daily session composed of 56 trials (~1-2 hours). At the beginning of each session, the house light remained off for an inter-trial interval (ITI), randomly chosen from an array (30, 60, or 90 s). After the ITI had elapsed, the house light turned on, signaling the start of the trial. During a trial, responses were reinforced on a fixed interval 30-s schedule of reinforcement; that is, operant responses emitted within the first 30 s of the trial were non-reinforced, and the first response that occurred 30 s after trial onset was reinforced by activation of the dipper. After the reinforcement, the house light was turned off, and the onset of a randomly selected ITI occurred prior to the next trial. A 30-s limited hold was instated, such that water delivery only occurred if the animal responded with a lever press between 30-60 s after onset of the house light illumination; the trial was terminated if no response was recorded within 60 s. After each FI training session, animals were allowed one hour of free access to water in their home cages, followed by the restricted access regime.

Peak Interval Training (10-20 sessions)

Animals were placed in the operant chambers for a total of 61 trials during the peak interval (PI) training. PI training sessions were conducted in order to reveal temporal control of behavior that is independent of motivational factors (Roberts, 1981). PI training included a combination of FI trials, which were identical to those in the previous phase, along with non-reinforced PI trials. Trials (FI or PI) were randomly chosen from seven blocks, each composed of 8 trials. There were 6 FI trials and 2 PI trials within each block. Following an FI or PI trial, the house light was
terminated for a randomly selected ITI duration (30, 60, or 90 s). PI trials were used to evaluate the animal’s acquisition of a memory for the 30-second no-response interval. Non-reinforced PI trials were identical to FI trials, except that the house light was illuminated for 90 s and operant responses were always non-reinforced. During this time, lever pressing was recorded. Response rates for individual animals during PI trials were analyzed daily to monitor distribution of lever pressing over the 90-second trial duration. Animals within a cohort were exposed to the same number of PI sessions until all animals within the cohort showed evidence of temporal control, i.e., a unimodal distribution of maximum lever pressing centered at around 30 s. At the end of the PI training phase, the water restriction schedule was terminated. It was reinstated during testing phases.

Peak Interval Probe Trial Test (5 sessions total per rat)

PI probe testing was conducted at different ages depending on the cohort (Cohort 1 = 12, 16, 19, and 24 months; Cohort 2 = 6, 9, 13, 18, and 21 months; Cohort 3 = 9, 12, 15, and 21 months). Similar to PI training, PI probe testing used 61 trials with the same ratio of reinforced to non-reinforced trials (i.e., 6 FI trials and 2 PI trials, respectively). Sessions began with a randomly chosen ITI (30, 60, or 90 s) followed by a FI 30 s trial. Probe trials were similar to FI trials except that 1) lever presses between 30 - 60 s did not result in water delivery and 2) the house light remained on for 90 s (i.e., three times the criterion duration). The distinction between PI training and PI probe testing was the number of sessions conducted and the age of the animals (young adult versus adulthood).
Behavioral data analysis

Data were collected and analyzed from the 5 sessions of peak interval probe tests per rat. Response rates were sorted into 1-second bins from the 1st second to the 90th second to determine the “peak response rate” and “peak response time.” The response rates for each animal were collected during each of the 90 bins during the session. Following this procedure, an average response rate per bin across trials was calculated for each rat. The bin in which the maximum rate of responding occurred was identified as the “peak response time”, and the rate of responding in that bin was the “peak response rate.” The peak response time ranged from 22-44 seconds, with a high number occurring during the 30-31 bin. The peak response rate ranged from 0.16 – 0.71 lever presses per second.

We used 2 measures as proxies for the “strength” of the memory for the time of reinforcement: “Accuracy” and “Precision.” Accuracy refers to the absolute difference between the average peak response time and the criterion time (i.e., 30 seconds) across all sessions. Precision measures stimulus control of behavior by time. In other words, it measures the degree to which lever pressing changes across the interval and can be thought of as response intensity--as a measure of the animal’s certainty. Our measure of precision was calculated by dividing the peak response rate by the average rate of responding during the 90-second duration. A ratio equal to 1 indicated a lack of temporally controlled responding, or lack of precision. In other words, the rate of responding was the same throughout the 90 seconds, with no indication of a memory of the time of reinforcement. A ratio greater than 1 indicated that the rate of peak lever pressing was greater than average, indicating that the rat increased lever pressing, which presumably reflects a memory of the time of reward.
In order to determine whether there were differences in levels of overall responding we measured “Average Response Rate” per bin across trials for each rat.

Immunohistochemistry

Animals were perfused at the ages of 24 months (cohort 1) or 21 months (cohort 2 and 3). Animals were perfused with 100 ml of 0.1 M phosphate buffered saline (PBS, pH 7.4) followed by 150 ml of 4% paraformaldehyde (pH 7.4). The brains were post-fixed in the same 4% paraformaldehyde solution for 1 hour, and then washed in PBS. Brains were stored overnight at 34°C in fresh PBS, then dehydrated in increasing ethanol concentrations (50, 70, 95 and 100%), and then embedded in polyethylene glycol (PEG; Polysciences). Ten-micron coronal sections in left and right hemispheres were cut via a rotary microtome, and every eighth section was mounted onto Superfrost+ slides in three series, air-dried overnight and stored at -20°C.

Three weeks after perfusions, we used immunohistochemistry (IHC) to label doublecortin (DCX), a microtubule-associated protein expressed in newly formed immature neurons. IHC batches were run with 1-2 slides from all animals so that tgHD and WT tissue was processed together. Slides were washed in tris buffered saline (TBS) for 10 minutes followed by a 30-minute incubation in a solution of 0.2% hydrogen peroxide in TBS to block endogenous peroxidase activity. After three 5-minute TBS rinses, a blocking solution of 3% normal horse serum and 2.5% Triton-X in TBS was applied and allowed to incubate for 30 minutes. Then the slides were incubated in primary anti-doublecortin made in goat (1:150, Santa Cruz Biotechnology, Dallas, TX) in 3% block at 4° C for 24 hours.

Following three 5-minute TBS rinses, tissue was incubated in biotinylated horse anti-goat secondary antibody in TBS (1:200, Vector Laboratory, Burlingame, CA) for three hours. After the slides were rinsed in TBS three times for 5 minutes each, they were incubated in a solution of
avidin-biotin complex (ABC Elite Kit; Vector Laboratory, Burlingame, CA) for one hour following manufacturer’s instructions. Tissue was again rinsed in three 5-minute TBS washes followed by incubation in diaminobenzidine (DAB) solution (1 drop of buffer, 2 drops of DAB, 1 drop of hydrogen peroxide, 2 drops of nickel for every 2.5 mL of distilled water) for 30 minutes (Vector Laboratory, Burlingame, CA). After three 5-minute TBS rinses, sections were dehydrated as follows: 30 seconds in water, 1 minute in 50% ethanol, 3 minutes in 70% ethanol, 5 minutes in 95% ethanol, two 10 minute rinses in 100% ethanol, two 15 minute rinses in xylene. The sections were then coverslipped with Krystalon mounting medium (Harleco, EM Science, Gibbstown, NJ).

**Microscope analysis**

Data were collected without knowledge of treatment group or hemisphere. Hippocampal tracing, SVZ tracing, area measurements, and cell counts were performed using mapping software (Neurolucida; MicroBrightField) coupled to a light microscope (Olympus BX51). Boundaries of the hippocampus (Figure 2A) and the SVZ of the lateral ventricles (Figure 2B) were traced with dark-field optics based on contrasting neuronal density using 4x (0.1 numerical aperture). The sub-granular zone (SGZ) and hilus sub-regions of the hippocampus were also traced using separate contours via dark-field under 4x magnification. New neurons in the SGZ and hilus (Figure 2C) of the hippocampus, and the SVZ (Figure 2D), were marked using light-field and 60x or 100x magnification.

The hippocampal and SVZ area and new neuron numbers were collected for every section (8-12 sections per animal) in both left and right hemispheres. Estimates of total new
neurons per mm$^2$ were calculated by dividing the number of new neurons by the area of the subregions of the hippocampus (i.e., SGZ, hilus, and SGZ+hilus) or the volume of the SVZ.

*Laterization index*

To compare the degree to which new neurons were laterализed towards either the left or right hemisphere, we calculated the Lateralization Index (LI) following Tsoi et al., (2014). The LI was calculated by subtracting the total density of new neurons in the right hemisphere from the left hemisphere and dividing that number by the mean density across both hemispheres. The LI index normalizes individual differences in overall numbers of new neurons to show the relative differences between hemispheres, independent of absolute number.

Lateralization index:

$$\frac{\text{Left neurons/mm}^2 - \text{Right neurons/mm}^2}{(\text{L neurons} + \text{R neurons}) / (\text{L area sampled} + \text{R area sampled})}$$

Positive values indicate higher new neuron density in the left hemisphere and negative values indicate higher new neuron density in the right hemisphere. The selection of which hemisphere was subtracted from the other, such that left-lateralized values were positive, was chosen to be consistent with the literature (Tsoi et al., 2014).

*Statistical Analysis*

Tukey’s post hoc tests were used following significant ANOVAs.
Cohort Effects

Timing behavior. We used a one factor ANOVA to determine whether there were differences in behavioral measures (i.e., accuracy, precision, average response rate) between the three cohorts. An independent t-test was also used to determine whether there were differences in timing behavior between rats sacrificed at age 24 months (cohorts 1) and rats sacrificed at 21 months (cohorts 2 and 3).

Age effect. We used a one factor ANOVA to evaluate whether there were behavioral differences between the time points that each cohort underwent testing (cohort 1 = 12 months, cohort 2 = 6 months, cohort 3 = 9 months).

New neurons. We used a one factor ANOVA to determine whether there were differences in number of new neurons in the left, right, and combined hemispheres of the hippocampal subregions (SGZ, hilus, and SGZ+hilus) across the three cohorts. We also ran a one factor ANOVA to determine whether there were differences in number of new neurons in the left, right, and combined hemispheres of the SVZ across the three cohorts.

Timing behavior

We ran unpaired t-tests to determine whether there were differences in timing behavior (accuracy, precision, average response rate) between treatment groups (WT vs tgHD).

New Neurons in the Hippocampus and SVZ

Comparing new neurons between genotypes

We ran an unpaired t-test to determine whether there were differences in mean density of new neurons in the SGZ, hilus, SGZ+hilus, and SVZ between treatment groups.
Comparing new neurons between hemispheres

We used a paired t-test to compare the numbers of new neurons between the left and right hemispheres of the SGZ, hilus, SGZ+hilus, and SVZ within treatment groups and across both groups combined. For all t-tests we used Bonferroni corrections for multiple comparisons. We also ran a 2 factor ANOVA with repeated measure on subregion to compare the difference in new neuron numbers between the hippocampal subregions (SGZ vs. hilus) of both treatment groups.

Comparing new neurons across subregions

A 2 factor ANOVA with repeated measure on hippocampal subregions region (SGZ vs hilus) was used to measure differences between hippocampal subregions that receive new neurons within and between treatment groups.

Timing Behavior and New Neurons in the Hippocampus and SVZ

SGZ, hilus, SGZ+hilus, and SVZ

We ran multiple regressions to determine the correlation between density of new neurons in the SGZ, hilus, SGZ+hilus and timing measures (accuracy, precision, average response rate).

Lateralization and timing behavior (accuracy, precision, average response rate). We used multiple regression analysis to determine whether the left-lateralization of new neurons in the SGZ, hilus, SGZ+hilus, and the SVZ was correlated with timing measures.
New Neurons in the Proliferative Zones: SGZ and SVZ

A 2 factor ANOVA with repeated measure on region was used to measure differences between regions that receive new neurons (i.e., SGZ and SVZ) within and between treatment groups.

Outliers

Grubbs’ test for outliers was used to determine whether there were significant differences in the mean number of neurons per animal within and between treatment groups (tgHD vs. WT). One rat in the WT group was removed from all analyses (p > 0.05).

Results

Cohort Effects

Timing behavior

We first assessed whether there were behavioral differences across the three cohorts in three behavioral measures: accuracy, precision, and average response rate. We found no significant differences between any of the behavioral measures (one factor ANOVA, p > 0.05 for all). Next, we compared whether there were behavioral differences (accuracy, precision, and average response rate) across the three WT cohorts and across the three tgHD cohorts. We found no significant differences between any of the behavioral measures within genotypes (one factor ANOVA, p > 0.05 for all).

Age effect

We also evaluated whether there were behavioral differences between cohorts 1, 2 and 3, which underwent testing at 12 months, 6 months, and 9 months, respectively. We did not find a
significant age effect in any of the behavioral measures (accuracy, precision, and average response rate; t-tests for all, p > 0.05). We assessed whether behavioral differences between cohorts 1, 2, and 3 differed across WT and tgHD groups. We found no significant differences in age of testing within genotypes (one factor ANOVA, p > 0.05 for all).

New neurons
No differences were found in the numbers of new neurons in the left, right, or combined hemisphere of the hippocampal subregions (SGZ, hilus, and SGZ+hilus) or SVZ across the three cohorts (one factor ANOVAs, p > 0.05 for all). We also assessed whether there were differences in new neuron numbers in the hippocampal subregions and SVZ across cohorts of each genotype (WT vs. tgHD). Again, there were no significant differences in new neuron numbers in each region within each genotype (one factor ANOVA, p > 0.05 for all). We also tested whether there was an age-effect in numbers of new neurons in the hippocampal subregions (SGZ, hilus, and SGZ+hilus) and SVZ between rats sacrificed at age 24 months (cohorts 1) and rats sacrificed at 21 months (cohorts 2 and 3) and found no significant differences (t-tests, p > 0.05). We also did not find age-effect in numbers of new neurons within genotypes (t-tests, p > 0.05). Given the lack of differences among cohorts, or between survival times, data from the 3 cohorts were combined for subsequent analyses.

Timing behavior
There was no difference in timing behavior (accuracy, precision, average response rate) between WT and tgHD groups (t-test, p > 0.05).
New Neurons in the Hippocampus

Table 1. Comparing new neurons between genotypes

<table>
<thead>
<tr>
<th>Hemisphere</th>
<th>LEFT</th>
<th>RIGHT</th>
<th>COMBINED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (mean +/- SEM)</td>
<td>tgHD (mean +/- SEM)</td>
<td>(t), P value</td>
</tr>
<tr>
<td>Region</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGZ</td>
<td>45.76 +/- 4.69</td>
<td>45.63 +/- 3.36</td>
<td>(-0.03), p = 0.97</td>
</tr>
<tr>
<td>Hilus</td>
<td>54.08 +/- 2.69</td>
<td>48.86 +/- 2.83</td>
<td>-1.58, p = 0.14</td>
</tr>
<tr>
<td>SGZ+Hilus</td>
<td>100.30 +/- 3.90</td>
<td>94.24 +/- 4.09</td>
<td>-1.27, p = 0.23</td>
</tr>
</tbody>
</table>

Bonferroni corrected values demonstrate that there were no group differences in mean density of new neurons in left, right, or combined hemispheres of any hippocampal region measured (unpaired t-tests, p > 0.05).

Comparing new neurons between hemispheres

There were no differences in the numbers of new neurons between the left and right hemispheres of the SGZ, hilus, SGZ+hilus either within each treatment group (WT or tgHD), or in both treatment groups combined (paired t-test, p > 0.05).

We also determined whether numbers of new neurons in the SGZ, hilus, or SGZ+hilus co-varied across hemispheres within individuals. We found a correlation between the numbers of new neurons in left and right SGZ in WT (r = 0.6, p = 0.04, Fig. 3A) and tgHD rats (r = 0.68, p = 0.02, Fig. 3B). However, there was no correlation between numbers of new neurons in the left and right hilus in either WT or tgHD rats (p > 0.05 for both). Interestingly, there was a significant positive correlation between numbers of new neurons in left and right SGZ+hilus in tgHD rats (r = 0.67, p = 0.02, Fig. 3B), but not WT rats (p > 0.05).
Comparing new neurons across subregions

However, when we compared new neuron numbers between the two hippocampal subregions (SGZ vs. hilus) in both treatment group, we found a main effect of subregion in the right hemisphere (2 factor ANOVA, repeated measure on subregion, \( F = 6.48, p = 0.02 \), Fig 4B) and combined hemispheres (\( F = 5.11, p = 0.04 \), Fig 4C), but no main effect in the left hemisphere (\( p = 0.11 \) Fig 4A). There was a greater density of new neurons in the hilus than the SGZ in the right hemisphere and combined hemispheres regardless of treatment condition. There was no main effect of treatment or interaction (\( p > 0.05 \)).

Timing Behavior and New Neurons in the Hippocampus

Next, we investigated the numbers of new neurons within the hippocampal subregions (SGZ, hilus, and SGZ+hilus) and timing behaviors (accuracy, precision, average response rate) to examine the potential relationship between these measures within both WT and tgHD rats.

SGZ

In WT rats, there were no significant correlations between timing behaviors (accuracy, precision, average response rate) and numbers of new neurons in the SGZ (\( p > 0.05 \) for all). Similarly, there was no relationship between timing behaviors and number of new neurons in the SGZ of tgHD rats (\( p > 0.05 \) for all, data not shown). Graphs are only presented for significant findings.

Hilus

In WT rats, there was a negative relationship between precision of timing behavior and numbers of new neurons in the right hilus (\( r = 0.66; p = 0.02 \), Fig 5A), a trend in combined hemispheres.
of the hilus \((r = 0.47; p = 0.08, \text{Fig} \ 5A)\), but no relationship in the left hilus \((p > 0.05)\), suggesting that more new neurons in the right hilus is associated with worse precision in WT rats. There was no relationship between new neuron density and other timing measures (accuracy and average response rate) in WT rats.

In tgHD rats, there was a positive relationship between precision and number of new neurons in the right hilus \((r = 0.59 \ p = 0.04, \text{Fig} \ 5B)\), and in combined hemispheres of the hilus \((r = 0.57; p = 0.04, \text{Fig} \ 5B)\), but no relationship was found in the left hilus \((p > 0.05)\). Furthermore, there was no relationship between number of new neurons and accuracy or average response rate in tgHD rats \((p > 0.05 \text{ for both})\).

\textit{SGZ+hilus}

In WT rats, there was a negative correlation between precision of timing and new neurons in the right SGZ+hilus \((r = 0.60; p = 0.04, \text{Fig} \ 6A)\), combined hemispheres of the SGZ+hilus \((r = 0.70; p = 0.01, \text{Fig} \ 6A)\), but no relationship in the left SGZ+hilus \((p > 0.05)\).

In tgHD rats, there were significant positive relationships between precision of timing and new neurons in the left SGZ+hilus \((r = 0.62, p = 0.03, \text{Fig} \ 6B)\), a trend in combined SGZ+hilus \((r = 0.51, p = 0.07, \text{Fig} \ 6B)\), but no relationship in the right SGZ+hilus \((p > 0.05)\).

This suggests that precision is correlated with the density of new neurons in the left hemisphere and combined hemispheres of the SGZ+hilus in tgHD rats, and an inverse relationship exists in WT rats. There was no relationship between new neuron density and other timing measures (accuracy and average response rate) in tgHD rats \((p > 0.05 \text{ for all})\).
**Lateralization**

Given the significant positive correlations found between new neurons and precision of timing behavior in the SGZ+hilus of tgHD rats, but not WT rats, we also investigated whether the degree of new neuron lateralization is correlated with accuracy, precision, or average response rate.

We found that in WT rats, precision was positively correlated with left side lateralization of new neurons in the hilus ($r = 0.65$, $p = 0.02$, Fig. 7A), but not in the SGZ or SGZ+hilus ($p > 0.05$ for both, Fig. 7A), suggesting that left side lateralization of new neurons in the hilus is associated with better precision in WT rats. There were no correlations between number of new neurons and accuracy or average response rates in WT rats ($p > 0.05$ for both).

In the tgHD rats, there were no significant correlations found between precision and the degree of new neuron lateralization in any subregions of the hippocampus ($p > 0.05$, Fig. 7B). Similarly, there was no relationship between lateralization of new neurons and accuracy or precision ($p > 0.05$ for both).

**New Neurons in the proliferative zones: SGZ and SVZ**

We compared numbers of new neurons between the neurogenic regions of the SGZ and SVZ in both treatment groups and found a significant main effect of region in the left hemisphere (2 factor ANOVA, repeated measure on region, $F = 9.17$, $p = 0.01$, Fig 8A), right hemisphere ($F = 23.23$, $p = 0.0007$, Fig 8B), and combined hemispheres ($F = 15.23$, $p = 0.002$, Fig 8C), suggesting that there is a significantly greater density of new neurons in the SVZ than the SGZ. There was no treatment effect and no significant interaction ($p > 0.05$).
New Neurons in the Subventricular Zone (SVZ):

Comparing new neurons between genotypes

We found no overall difference in numbers of new neurons in the left, right, and combined hemispheres of the SVZ between WT and tgHD groups (t-test, p > 0.05).

Comparing new neurons between hemispheres

We measured differences between hemispheres by treatment condition and again found no significant differences or interaction (2 factor ANOVA, treatment and hemispheres, repeated measure on hemispheres, p > 0.05). There were no differences in the numbers of new neurons between the left and right hemispheres either within treatment groups, or across both groups combined (paired t-test, p > 0.05).

We also determined whether numbers of new neurons in the SVZ were correlated across hemispheres. We found a trend towards a positive correlation between the numbers of new neurons in the left and right SVZ in tgHD rats (r = 0.49, p = 0.07, Fig 9B) but no correlation in the left and right SVZ in WT rats (r = 0.002, p > 0.05, Fig 9A).

Timing Behavior and New Neurons in the SVZ

Next, we investigated the relationship between the numbers of new neurons in the SVZ and timing behavior (accuracy, precision, average response rate) to examine the potential relationship between these measures within both WT and tgHD rats. We found that in WT and tgHD rats, there was no correlation between number of new neurons in the left, right, or combined hemispheres of the SVZ and accuracy of timing (p > 0.05 for all). There was a significant negative correlation between precision of timing and number of new neurons in combined hemispheres of the SVZ (r = 0.8, p = 0.04, Fig. 10A) in WT rats, but this relationship was not
found in the left hemisphere or right hemispheres of the SVZ. No relationship was found between number of new neurons and precision of timing in the left, right, or combined hemispheres of the SVZ (p > 0.05 for all, Fig. 10B) in tgHD rats. There was no relationship between the number of new neurons in the SVZ and average response rate in WT or tgHD rats (p > 0.05 for all).

**Discussion**

New neurons in the SGZ of the dentate gyrus in the hippocampus and the SVZ of the lateral ventricles are thought to play a role in plasticity and learning (Deng et al., 2010; Kempermann et al., 1997; van Praag et al., 1999; Jessberger et al., 2009; Saxe et al., 2006; Clelland et al., 2009; Sahay et al., 2011). In neurodegenerative diseases, such as Huntington’s disease, pathophysiological features include significant atrophy and alteration of neurogenesis in both the hippocampus and the striatum (i.e., one of the target migration regions of new neurons in the SVZ). In the present study, we expected that tgHD rats would perform worse on timing behaviors that underlie both the hippocampus and striatum. Instead, we found that WT rats and tgHD rats performed similarly on timing tasks. Interestingly, we discovered that greater numbers of new neurons in the right and combined hemispheres of hippocampal subregions (SGZ+hilus) were associated with worse timing behavior in WT rats, and greater number of new neurons in the left and combined hemispheres of hippocampal subregions (SGZ+hilus) were associated with improved timing behavior in tgHD rats. We found a similar pattern in the SVZ, whereby greater numbers of new neurons were correlated with worse timing performance in WT rats. There was, however, no relationship between number of new neurons in the SVZ and timing performance in tgHD rats. The relationship between performance on timing tasks and number of new neurons
suggests that new neurons in the hippocampus and SVZ adversely effects timing performance in WT rats while improving timing performance in the hippocampus of tgHD rats.

We also found that the degree of left-side lateralization of new neurons, which is the *relative* difference of the number of new neurons between the left and the right hemispheres, was positively correlated with timing behavior in both WT and tgHD rats. Specifically, left-side lateralization of new neurons in the hilus subregion of the hippocampus was associated with improved timing in WT rats. The left-side lateralization of new neurons in the SVZ was associated with better timing in tgHD rats. The correlation between timing performance and left-lateralization suggests not only a potential benefit for greater numbers of new neurons in the left hippocampus of WT rats and left SVZ of tgHD rats, but also a disadvantage to having new neurons in the right hemispheres of these regions.

**Time cells in the hippocampus**

The hippocampus plays an essential role in the temporal organization of memories (Eichenbaum, 2014). One of the mechanisms for this temporal organization are hippocampal “time cells,” which are neurons in the hippocampal area CA1 that fire at distinct moments in temporally structured experiences (Pastalkova et al., 2008; MacDonald et al., 2011, 2013; Kraus et al., 2013). “Time cells” have been recorded from left (MacDonald et al., 2013; Manns et al., 2007) and bilateral CA1 (Kraus et al., 2013), and have more recently been found in CA3 (Salz et al., 2016). Both CA1 and CA3 receive ipsilateral and contralateral projections from the dentate gyrus. See Buzsaki and Tingley (2018) for an alternative concept of temporal processing.
New neurons are detrimental to timing performance in WT rats

*Hippocampus*

We found that greater numbers of new neurons in the hippocampus of WT rats were correlated with worse performance on a timing task. This finding is not entirely surprising in light of previous research suggesting that new neurons may potentially alter hippocampal circuits, leading to cognitive impairment (Akers et al., 2014; Frankland et al., 2013). Martinez-Canabal (2012) found that mice with increased number of granule cells in the hippocampus had impaired spatial learning. One idea explaining the relationship between a greater number of neurons and impaired performance is that the quiescence of dentate granule cells in the hippocampus ensures that the dentate gyrus relays a sparse code onto target cells in the CA3 region of the hippocampus. This sparse coding is thought to be critical for memory formation, and particularly beneficial for pattern separation (Treves and Rolls, 1994). Thus, increasing the number of granule cells may compromise sparse coding and the ability of the dentate gyrus-CA3 to pattern-separate.

To our knowledge, no previous studies have examined neurogenesis and timing behaviors. Interestingly, manipulations that increase neurogenesis may have positive effects on some behaviors, but negative effects on others. For example, new neurons have a positive effect on trace and contextual fear conditioning (Gould et al., 1999), but not on spatial learning (Van der Borght, et al., 2005). Animals without new neurons also perform better in certain working memory paradigms, as well as on tasks with repetitive information (Scharfman and Hen, 2007). These findings demonstrate that adult born neurons make a distinct contribution to some but not all hippocampal functions.
Reasons for the distinction between the roles of new neurons in contextual fear conditioning versus spatial maze learning may include the different molecular signaling pathways within the hippocampus used by each learning task. Also, new neurons may influence learning and memory primarily in tasks that involve emotional arousal or co-activation of the hippocampus and the amygdala. Finally, exposure to novel environments increases GABAergic tone in the dentate gyrus and facilitates the generation of long-term potentiation (Saxe et al., 2006). Since young neurons are not inhibited by GABA, they may be preferentially recruited under such conditions (i.e., novel environments). If so, the effects of blocking neurogenesis would be most impactful in tasks that take place in a novel environment. This would explain why decreased numbers of new neurons impair context conditioning, but not water maze performance because contextual conditioning occurs in a single exposure to a novel environment, whereas learning in the water maze requires repeated exposure to an environment over multiple days. Lastly, Frankland (2013) proposed a clearance mechanism, by which new cell integration results in a remodeling of circuitry that clears memories from the hippocampus. This remodeling of circuitry may either degrade memories already stored in those circuits, or may make those memories increasingly difficult to access. Akers et al (2014) demonstrated that voluntary exercise increased hippocampal neurogenesis in adult mice, which led to forgetting of established contextual fear and spatial memories. Furthermore, pharmacological (e.g., memantine, fluoxetine) and genetic (deletion of p53 from neural progenitors) manipulations that artificially elevate hippocampal neurogenesis when introduced after training, similarly weakened existing hippocampus-dependent memories. This suggests that exercise-induced forgetting is mediated by a neurogenesis mechanism.
Taken together, potential reasons as to why a negative, rather than a positive, relationship may exist between the number of new neurons in the hippocampus and performance on a timing task include compromised sparse coding in the dentate gyrus, the specific molecular signaling pathway used by the timing task, the co-activation of other brain regions required to perform the task, repeated exposure to an environment over multiple days, and a remodeling of hippocampal circuitry.

**SVZ**

We found that an increase in new neurons in the SVZ of WT rats corresponded to worse timing performance. Reasons for this negative correlation may in part be related to new neurons in the SVZ causing disruptions in the already established circuits of the striatum. However, an experiment testing this idea has yet to be carried out. Given that the striatum underlies timing behavior, we expect that the negative correlation found in the present experiment is being driven by the new neurons in the SVZ that will eventually reside in the striatum rather than the olfactory bulb. However, it is important to note that in the present study, the negative correlation between neurogenesis and timing behavior in the SVZ may be a spurious finding due to the small sample size (n = 5) and only two data points that are driving this significant relationship.

Previous research has suggested that striatal tumors and ischemia attract precursor cells away from the SVZ and towards the striatum (Glass et al., 2005). In the present study, we did not investigate whether rats had developed an additional disease process (i.e., tumor or ischemia) that may have impacted the striatum, and thus affected timing performance. If, in fact, the striatum of WT rats were affected, it would explain the reason why a greater number of new neurons would be correlated with worse performance on a timing task.
Information on striatal new neurons is limited to morphological and neurochemical descriptions (Tepper et al., 2010). Currently, the functional purpose of new neurons in the striatum remains a mystery. Therefore, we can currently only speculate about the potential function of continuous striatal neurogenesis in mammals, and the functional integration of new neurons into existing neuronal circuits.

New neurons are beneficial to timing performance in diseased brains

**Hippocampus**

The mammalian brain displays an inherent capacity for functional homeostasis by using compensatory mechanisms that counteract injury or disease-induced changes as an attempt to preserve adequate brain function (Fornito et al., 2015; Caleo, 2015; Harel and Strittmatter, 2006). Given its capacity to attempt restoration of function, the relationship between number of new neurons and improved timing performance in tgHD rats is unsurprising. In line with these findings, it was previously discovered that enhancement of endogenous neural stem cells in the hippocampus abolishes hippocampal-dependent memory deficits in a mouse model of Alzheimer’s disease (Richetin et al., 2014). In HD animal models, enhanced neurogenesis in the hippocampus as a result of environmental enrichment (Lazic et al., 2006) and voluntary physical exercise (Pang et al., 2006; van Dellen et al., 2000) have been shown to either attenuate motor dysfunction (Hockly 2002; Schilling et al., 2004; van Dellen, 2000), reduce cognitive impairment (Pang et al., 2006), or increase survival rates (Hockly et al., 2002; Bjugstad et al., 2001; Zhou and DiFiglia, 1993). Thus, there may be a compensatory mechanism underlying the relationship between greater number of new neurons and improved performance on a timing task in tgHD rats.
Left-sided lateralization of new neurons improves behavior

Here we demonstrated that left-side lateralization of new neurons in a hippocampal region (i.e., hilus) is associated with improved timing performance in WT rats. It is well established that memory and spatial navigation are lateralized in the human hippocampus. However, whether new neurons are similarly lateralized has never been explored in human or rodent research. Tsoi et al., (2014) found that lateralization of new neurons to the left hemisphere in zebra finches corresponded to the strength of the memory of their song, suggesting that a greater number of new neurons in the left hemisphere may be functionally related to better learning and memory.

A mechanistic explanation describing how left sided lateralization of new neurons may improve timing performance is unclear. However, if there is a functional relationship between these variables, it may be that new neurons allow for greater plasticity in the left hemisphere, which allows for increased information processing. On the other hand, the right hemisphere may possibly maintain previously learned information and thus benefits from fewer new neurons, resulting in a stability of circuits. Although the functional interaction between left and right hemispheres remains unclear, this is the first study to establish a relationship between lateralization in numbers of new neurons and timing behavior.

Limitations to the current study:

Validity of doublecortin as a marker of adult neurogenesis

Doublecortin (DCX) is a microtubule associated protein that plays an important role in the migration of immature neurons from their birthplace (i.e., ventricular zone in songbirds; SVZ and SGZ in mammals) to their final destination (Vellema, et al., 2014). Impairment of the X-linked gene DCX results in alterations of cortical laminar organization, suggesting that DCX is
vital to the proper migration of new neurons to the cortex (in mammals), or the pallium (in
gamebirds; Vellema, et al., 2014). Given these findings, doublecortin is a useful tool for studying
adult neurogenesis in comparative neurobiology (Balthazart & Ball, 2014), despite contentions
by Vellema and others that the marker is non-specific in the age of neurons it labels (Vellema, et
al., 2014). DCX is expressed by both migratory and mature, post-migratory neurons in the
vertebrate brain. However, it has also been found in regions that do not incorporate new neurons
in adulthood (Vellema, et al., 2014). This could suggest that DCX is involved in the maintenance
and alteration of the cytoskeleton in mature neurons, perhaps underlying neurite reorganization
and/or synaptogenesis, as has been shown in the rat brain (Brown, et al., 2003). Therefore, the
conclusions drawn from DCX-labeling are not always quite clear since it prevents us from
examining the exact birthdates of neurons and their subsequent survival. One way to bypass
these potential problems is by using the mitotic marker bromodeoxyuridine (BrdU).

Despite these concerns, comparisons between DCX-labeled cells and BrdU-labeled cells
in the SGZ have established DCX as an accurate marker of new neurons in this region (Rao &
Shetty, 2004).

Age of tgHD rats at the time of sacrifice
The lifespan of rats is approximately two years. At the time of sacrifice, tgHD rats in the present
study were either 21 or 24 months old. Although neurogenesis continues throughout life, its rate
does not decline with increasing age in rodents (Seki and Arai 1995; Kuhn et al., 1996; Kempermann et
al., 2002) and non-human primates (Gould et al., 1999). In older rats, the proliferation rate of
neural stem cells in the SGZ of the dentate gyrus is reduced by 80% (Jin et al., 2003), with the
majority of these age-related decreases in SGZ neurogenesis taking place between 3 and 12
months of age (Kuhn et al., 1996). The proportion of these neural stem cells that survive to become mature neuronal cells is reduced to half of that of young animals (Tang et al., 2007). In contrast, there are no significant decreases in the rate of neural stem cell proliferation in the SVZ (Jin et al., 2003). In light of these findings, it is important to consider that the rats used in the present study were nearing the end of their lifespan, and the relationship between new neurons and behavior may prove to be very different in younger rats.

Figure 1. The hippocampus. A) Regions of the hippocampus, including area CA1, which is a hippocampal sub-region primarily affected in Huntington’s disease (Zabenko & Pivneva, 2016). B) Structures of the hippocampus. Anatomically related structures include the parahippocampal cortex, entorhinal cortex, and perirhinal cortex.
**Figure 2.** A) Hippocampal subregions: subgranular zone (SGZ) and hilus (4x magnification). Scale bar, 1 mm. B) Subventricular zone (SVZ) and striatum (4x magnification). Scale bar, 1 mm. C) New neuron in the SGZ (60x magnification). Scale bar, 25 μm. D) New neuron in the SVZ (60x magnification). Scale bar, 25 μm.
There was a correlation between the density of new neurons in left and right SGZ in WT and tgHD rats. However, there was no correlation between numbers of new neurons in the left and right hilus in either WT or tgHD rats. Interestingly, there was a significant positive correlation between numbers of new neurons in left and right SGZ+hilus in tgHD rats, but not WT rats.

We found a main effect of subregion (SGZ and hilus) in the right hemisphere and combined hemispheres, but no difference between regions in the left hemisphere, suggesting that...
there is a greater density of new neurons in the hilus than the SGZ in the right hemisphere and combined hemispheres regardless of treatment condition.

Figure 5: (A) In WT animals, we found a significant negative relationship between precision and the number of new neurons in the right hilus, and a trend in both hemispheres combined, but no relationship in the left hilus. (B) In tgHD animals, we found a significant positive correlation between precision and the total number of new neurons in the right hilus and combined hemispheres of the hilus. However, there was no relationship between precision and number of new neurons in the left hilus of tgHD rats.
Figure 6: (A) In WT rats, we found a significant negative correlation between precision and density of new neurons in the right SGZ+hilus and combined hemispheres of the SGZ+hilus. There was no relationship between precision and density of new neurons in the left SGZ+hilus. (B) In tgHD rats, there was a significant positive correlation between precision of timing behavior and density of new neurons in the left SGZ+hilus and a trend towards a significant relationship between precision and density of new neurons in the combined hemispheres of the SGZ+hilus. There was no relationship between precision and density of new neurons in the right SGZ+hilus of tgHD rats.
Figure 7: Precision and lateralization of new neurons in (A) WT and (B) tgHD rats by hippocampal subregion (SGZ, hilus, SGZ+hilus). Higher positive values along the x-axis indicate more new neurons in the left hemisphere relative to the right. Negative numbers along the x-axis indicate more new neurons in the right hemisphere relative to the left. (A) In WT rats precision was positively correlated with left side lateralization of new neurons in the hilus. (B) In tgHD rats, there were no significant correlations found between precision and the degree of new neuron lateralization in hippocampal subregions (SGZ, hilus, SGZ+hilus).
Figure 8: We found a significant main effect of region (SGZ and SVZ) in the left hemisphere, right hemisphere, and combined hemispheres, suggesting that there is significantly greater density of new neurons in the left, right, and combined hemispheres of the SVZ compared to the SGZ.

Figure 9. There was no correlation between numbers of new neurons in left and right SVZ in WT rats and a trend towards a significant correlation in tgHD rats.
Figure 10: In WT rats, there was a significant negative correlation between precision of timing and number of new neurons in combined hemispheres of the SVZ, but this relationship was not found in the left hemisphere or right hemispheres of the SVZ. In tgHD rats, there was no relationship between precision of timing and number of new neurons in the left, right, or combined hemispheres of the SVZ.
CHAPTER 3: ALTERED SENSORY FEEDBACK IMPACTS NEW NEURONS IN A REGION AND HEMISPHERE-SPECIFIC MANNER

Why Songbirds?

Unlike all mammals excepts humans, songbirds have the ability to learn vocalizations, and lifelong addition of new neurons occurs in discrete nuclei that subserve song learning, production, and perception. The neural plasticity linked to a well-defined and quantifiable behavior makes the avian brain a valuable model for understanding factors that influence neuronal lifespan. An understanding of such factors may contribute to the development of interventions for improving the survival of new neurons generated post brain injury or during neurodegenerative disease, and also has the potential to inform targeted therapeutic strategies to promote brain health.

Development of Song in Zebra Finches

Adult male zebra finches (*Taeniopygia guttata*) produce a single, stereotyped song that they learn as juveniles by imitating an adult conspecific (i.e., a “tutor”; Doupe & Kuhl, 1999). Song learning occurs during an early critical period, known as the “sensory period”. In zebra finches, only the males sing. Juvenile male birds listen to adult songs and form a “template,” or an auditory memory, of the tutor’s song (Brainard & Doupe, 2000a). Songbirds that are deprived of a tutor during the sensory phase, but provided with a tutor at a later stage of development, produce aberrant songs with structural alterations (Wilbrecht et al., 2002). The subsequent stage of song development occurs during the “sensorimotor period,” in which juvenile birds compare expected and received auditory feedback during singing. This comparison is used to shape vocal
output of the bird’s own song until an accurate match is made between the bird’s song and the auditory template of the tutor’s song. Evidence for the importance of auditory feedback for song learning is seen in the abnormal songs produced by birds that have been deafened (Konishi, 1969). During the final stage of song learning and production (approximately 90 days post hatch), the song structure stabilizes in a process called “crystallization.” From this point forward, the bird reproduces the learned song with accuracy and precision throughout its entire life (Brainard & Doupe, 2000b).

After song learning is completed, slight discrepancies between expected and received auditory feedback may produce error signals that result in adjustments to motor commands in order to maintain a stereotyped song throughout adulthood. The idea that experience contributes to motor stability in postcrystallization singing would indicate that song development is a lifelong process, even in bird species with a song repertoire of only one song. Interestingly, Waldstein (1990) demonstrated that similar to human speech, the dependence of adult song on auditory feedback decreases with older age, suggesting that the motor process for song becomes increasingly stable with age, singing experience, or both (Lombardino and Nottebohm, 2000; Brainard and Doupe, 2001).

**Neuroanatomy of the Song System Pathways**

Two neural pathways work in conjunction to generate learned song in all songbirds. The production of song is driven by a direct vocal motor pathway (VMP): the telencephalic premotor nucleus HVC (a letter based name, formerly known as High Vocal Center) projects to the robust nucleus of the archistriatum (RA), which in turn projects to the motor neurons in the brain stem hypoglossal nucleus (nXII) that innervate the muscles of the syrinx, the bird’s vocal organ, via
the tracheosyringeal branch of the hypoglossal nerve (NXIIIts) (Figure 11). The pathway necessary for song acquisition by juveniles is driven by a “cortico”-thalamic-ganglia loop (Bottjer and Johnson, 1997; Reiner et al., 2004; Person et al., 2008) known as the anterior forebrain pathway (AFP): a discrete region in the medial striatum, Area X, receives input from the nucleus HVC and projects to the medial nucleus of the dorsolateral thalamus (DLM), which projects to the lateral magnocellular nucleus of the anterior nidopallium (IMAN). Cells in IMAN project back to Area X and also send axons to nucleus RA, a region responsible for song motor commands in the descending motor pathway (Figure 1).

The ascending auditory pathway comprises a higher order auditory region, caudomedial nidopallium (NCM), which has indirect projections to the vocal production system. The ascending auditory pathway in songbirds is analogous to that of mammals: the cochlear nucleus (CN) sends projections to the superior olive (SO), lateral lemniscus (LL), and MLd (a homolog of the inferior colliculus). MLd then projects to the thalamic auditory relay nucleus, ovoidalis (Ov), which in turn, projects to Field L, analogous to mammalian primary auditory cortex. Finally, higher order auditory areas such as the caudomedial mesopallium (CMM) and NCM receive input from Field L (Figure 1).

The VMP, AFP and the auditory pathway, are functionally-specialized independent yet integrated pathways that play important roles in song learning, production, and perception.

The Song System is Replicated in Both Hemispheres

The song system consists of two functional halves, each of which controls the ipsilateral half of the syrinx. Therefore, it is possible to peripherally disconnect one or both halves of the song system from their respective syringeal muscles by lesioning one or both tracheosyringeal nerves.
When one syringeal half is denervated, early in life, the bird is still able to imitate sounds with a hemi-syrinx. In adulthood, unilateral syringeal denervation distorts the normally stereotyped song structure and provides the animal with altered auditory and perhaps proprioceptive feedback. Thus unilateral denervation makes it possible to compare hemispheres that are or are not intact in syringeal control within an animal.

Nuclei within the Song System: HVC, Area X, and NCM Continuously Receive New Neurons

Although new neurons are found widely throughout the avian telencephalon, only three song system regions receive new neurons: HVC, Area X, and NCM. These regions exhibit large-scale neuron addition both after zebra finches hatch and throughout adulthood (Alvarez-Buylla and Kirn, 1997; Lipkind et al., 2002; Barnea et al., 2006; Barkan et al., 2007; Adar et al., 2008), although after song crystallization, rates of new neuron addition decline, and continue to decline as the bird ages (Dewulf & Bottjer, 2002).

**HVC**

HVC is a telencephalic premotor nucleus necessary for the production of learned song in all songbirds (Nottebohm, et al., 1976). Lesions to HVC result in an inability to produce song, however, birds are still able to produce “silent song,” which entails a singing posture with song-like beak movements (Nottebohm et al., 1976). Neurons within HVC show auditory responses to song playback from the bird’s own song, but interestingly, activity is inhibited during song production itself (Katz & Gurney, 1981). The diverse connections that HVC receives from areas that subserve sensorimotor and auditory processing (i.e., nucleus interfacialis of the nidopallium, field L, and magnocellular nucleus of anterior nidopallium), as well as the projections it sends to
regions underlying song learning and production (i.e., Area X and RA) suggests that HVC plays a role in integration of sensory and motor information (Margoliash, 1997).

**Area X**

Area X is a forebrain nucleus homologous to a combined striatum and putamen of the mammalian basal ganglia (Gale and Perkel, 2010), and is necessary for proper vocal learning in songbirds (Nottebohm et al., 1976). Lesions to Area X in juvenile birds result in song instability and prevent the normal development of a stereotyped adult song (Scharff and Nottebohm, 1991). Throughout the song learning process, neurons in Area X shift their auditory tuning from a preference for playback of the tutor’s song, to a preference for the bird’s own song (Solis and Doupe, 1999). This change in preference may indicate that Area X guides song learning by playing a role in processing auditory feedback during juvenile song production.

The development of the bird’s song becomes largely independent of auditory feedback in adulthood once the song motor pattern becomes “crystallized” at the end of the critical period for song learning (Price, 1979; Pytte and Suthers, 2000). After this time, bilateral lesions to Area X or lMAN do not produce noticeable effects in adult song structure (Bottjer, 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). Interestingly, however, the AFP continues to receive song feedback in adulthood (Doupe, 1997; Brainard and Doupe, 2000b; Person and Perkel, 2007; Prather et al., 2008) and induces song variability when auditory feedback is blocked or altered (Williams and Mehta, 1999; Brainard and Doupe, 2000a; Thompson et al., 2007; Nordeen and Nordeen, 2010). Thus, it is thought that the adult function of Area X is to correct small changes in song that result from passive processes (i.e. hearing loss, syringeal muscle/membrane changes) or from external damage to the motor system (Brainard and Doupe, 2000a). Area X
may directly contribute to this function by providing information about whether song feedback is accurate, i.e., matched to expected feedback (Brainard and Doupe, 2000b; Fee, 2011). Therefore, in addition to generating variability, the AFP is thought to evaluate vocal errors and transmit a signal to guide plasticity in the motor pathway (Brainard and Doupe, 2000b; Person et al., 2008; Andelman and Fee, 2009).

NCM

NCM is involved in auditory perception, specifically of learned vocalizations, as well as auditory memory, and is also thought to play a role in adult song maintenance and discrimination (Bolhuis and Gahr, 2006; Phan et al., 2006). This was demonstrated by a study that distorted self-generated auditory feedback by playing a loud white noise during singing (Canopoli et al., 2014), which consequently shifted song structure. A few days after the white noise playback ended, birds restored their original song. However, birds with bilateral lesions in NCM did not recover. This suggests that NCM may store a memory of the bird’s own song or the tutor’s song, either of which may be necessary to recover song production after singing is temporarily perturbed.

Neurons in NCM are more responsive to playback of the tutor’s song and bird’s own song than to songs of conspecifics (Margoliash, 1986; Voss et al., 2007). However, many NCM cells respond preferentially to the tutor song and not to the bird’s own song, and the strength of the response to the tutor song correlates with the degree to which birds have accurately copied the tutor song (Phan et al., 2006). Recent work indicates that disruption of immediate early gene (IEG) expression (a marker of neuronal activity) in NCM and surrounding auditory structures during song tutoring prevents young zebra finches from copying tutor song, without altering performance on auditory perception tasks (London and Clayton, 2008). This is consistent with
the idea that sensory template information is stored in NCM and related auditory structures, which relay information to HVC and the AFP where it is used to guide sensory-motor learning in young juveniles (Bolhuis and Gahr, 2006; Phan et al., 2006).

In addition to tutor song recognition, it appears that activity in NCM also underlies perceptual processing and memory of conspecific vocalizations, as measured by electrophysiology (Chew et al., 1995; Chew, et al., 1996; Phan, et al., 2006), expression of the immediate early gene Zenk (Bolhuis et al., 2000; Bolhuis et al., 2001; Huesmann et al., 2000) and behavioral recognition tasks (Gobes Bolhuis, 2007).

**New Neurons in HVC**

Factors that promote new neurons in the songbird have been studied most systematically in HVC. New neurons are produced within the subventricular zone of the lateral ventricles and migrate a short distance into HVC, where they extend axons to the pre-motor robust nucleus of RA, becoming part of the vocal motor pathway. New neurons start arriving in HVC by 1-2 weeks post-birth (Kirn, et al., 1999) with some new cells dying during migration (Alvarez-Buylla and Nottebohm, 1998), and half of the cohort having died by week 3 (Kirn et al., 1999). The amount of singing has been shown to correspond to new neuron survival (Li et al., 2000, Alvarez-Borda and Nottebohm, 2002.), and this is mediated by BDNF and testosterone, both of which also have a direct impact on new neuron survival regardless of the animal’s behavior (Brenowitz 2015; Rasika et al., 1999, Rasika et al., 1994; Alvarez-Borda and Nottebohm, 2002; Alward et al., 2016). By the same token, birds that are prevented from singing show decreased survival of new neurons in HVC (Li, et al., 2000). Thus, the prevailing idea is that neuronal
activity confers survival (Larson et al., 2013; Brenowitz, 2015; Pfisterer and Khodosevich, 2017; Hall and Tropepe, 2018; Alvarez-Buylla, 2002; Kempermann et al., 1998; Ninkovic et al., 2007).

Housing conditions also impact the lifespan of HVC neurons, with more new neurons surviving to 1 month post birthdating in zebra finches housed in a group compared to singly housed birds, with no group differences in proliferation (Walton et al., 2012). Group housing likely results in both increased singing and increased auditory exposure to song, each a likely candidate for enhancing neuronal survival by increasing neuronal activity.

*New Neurons in Area X*

In contrast to HVC, neuron addition to Area X in relation to singing behavior has been much less explored. However, it is known that the age-related increases in song stereotypy reported in adult zebra finches are not associated with changes in Area X neuron addition, suggesting that the functional purpose of new neurons in Area X differs from that of neurons added to HVC (Pytte et al., 2007). Between post hatch days 25-50, Area X doubles in size, and similar to HVC, much of this growth is due to neuron addition (Kirn and DeVoogd, 1989; Burek et al., 1991). However, unlike in HVC, Area X cell addition over this time appears to be restricted to interneurons (Sohrabji et al., 1993).

*New Neurons in NCM*

Blocking auditory input into NCM in adulthood by deafening decreased new neuron survival in NCM. This supports the idea that new neuron survival may be linked to functional activity (Pytte, et al., 2011). Furthermore, Tsoi et al (2014) discovered that more new neurons were added to left NCM than right NCM in adult birds. The degree of asymmetry in new neuron
incorporation is correlated with both the quality of song imitation and the strength of neuronal memories for recently heard conspecific songs. These correlations suggest that the relative differences in new neurons between hemispheres are an important factor in song learning and memory processes.

The location of a neuron in NCM can determine for how long it will survive. Barnea et al., (2006) found that different parts of the brain may upgrade memories at different time intervals, resulting in an anatomical representation of time in the brain. For instance, there was quicker turnover of new neurons in the caudal part of NCM than in the rostral area, suggesting that the updating of information might occur at different intervals in different parts of NCM. Following this study, it was also found that a neuron’s age at the time that a zebra finch is exposed to environmental change can also determine its survival in NCM (Adar et al., 2008). If the bird was introduced into a novel social setting, neurons that were 1-month old at this time survived better than neurons that were 3-months old at the same time, and these effects were dependent on their position within NCM. This finding suggests that the location of a neuron within NCM, combined with the age of the neuron as it acquires new information, will impact its survival.

Present Investigation

Pytte et al., (2008) proposed that the lifespan of new neurons is prolonged when they make successful contributions to song performance. This model is supported by the association between numbers of new neurons added to HVC and the quality of song structure, suggesting that song stereotypy is achieved when “incorrect” neurons in HVC are shed and replaced with
“correct” neurons that contribute to the song circuitry, thereby prolonging their survival. This pattern suggests that new neurons may be added and actively maintained in concert with song quality (Pytte et al., 2008). Quality, as used here, is a measure of a target, goal, or “correct” song.

To test this idea, Pytte et al., (2011) experimentally altered song quality, creating aberrant songs by partially and reversibly paralyzing the vocal syringeal muscles with injections of botox (Pytte et al., 2011; Pytte and Suthers 2000). They found a positive correlation between the degree of recovery toward the bird’s original song, and numbers of new neurons in HVC. Increasingly intact songs corresponded to higher numbers of new neurons in the song production pathway. However, due to the temporary effects of the syringeal paralysis, the directionality of this correlation was ambiguous. It was not clear whether the quality of song structure was the cause of, or resulted from, increased new neuron addition to HVC. To resolve this ambiguity, we “clamped” aberrant song structure, holding it constant, which was not done in Pytte (2011). This allowed us to examine neurogenesis in the presence of stable, aberrant auditory feedback.

To do this, we unilaterally denervated the syrinx in adult male zebra finches to produce an irreversible disrupted song, resulting in altered auditory and sensorimotor feedback during song production. This allowed us to determine whether song quality impacts numbers of new neurons in HVC, Area X, and NCM. Given the connections between the three regions, and their involvement in song behavior, we took advantage of the whole brain and song system to explore effects of altered sensory feedback on new neurons across regions and hemispheres. An understanding of how the process of neurogenesis is impacted within and across brain regions may have the potential of informing strategies that consider a whole brain system to help promote neurogenesis.
Materials and Methods

Animals

All methods were approved by the Queens College Institutional Animal Care and Use Committee (IACUC). Subjects were adult male zebra finches between 4 and 11 months of age (n = 49). Birds were hatched in either the Queens College or Wesleyan University breeding colony, where they were kept with their parents until 90 days of age, which is considered adulthood. Thereafter, birds were housed in group cages within auditory and visual contact of both sexes until the time of sacrifice. Birds were maintained on a 12:12 h light:dark schedule. Sample sizes for groups processed to label DCX were: left nXIIIts-cut (n = 10), right nXIIIts-cut (n = 9), sham (n = 8). Sample sizes for groups processed to label BrdU/Hu in HVC and Area X were left nXIIIts-cut (n = 14), right nXIIIts-cut (n = 13), sham (n = 12). For labeling BrdU/Hu in NCM, group sizes were left nXIIIts-cut (n = 17), right nXIIIts-cut (n = 15), and sham (n = 17). Variability in sample sizes was due to tissue damage in the nuclei of interest during tissue processing.

Song Recording and Analysis

For song recording, adult male zebra finches were housed individually in sound attenuated chambers where they were recorded continuously for 2 - 4 days prior to nXIIIts cut, and again 1 - 2 days after surgery (Earthworks precision audio SR20 Cardioid Microphone) using sound-activated Avisoft Recorder (Avisoft Bioacoustics). The 10 highest quality pre and post-operative songs were edited to single motifs, i.e., “songs” or long stereotyped sequences of sounds, with Raven sound analysis software (Cornell Lab of Ornithology). Song stereotypy and changes in structure after nXIIIts-cut were computed using Sound Analysis Pro (Tchernichovski, et al., 2000), which calculates and compares the structure of sound pairs based on measurements of
pitch, frequency modulation, amplitude modulation, Wiener entropy (noisiness), and goodness of pitch (how periodic or harmonic the acoustic structure is). We used two measures to assess pre- and post-operative song quality: (1) percentage of similarity (termed here “similarity”) and (2) accuracy. Similarity is the percentage of pre-operative songs that meet an experimenter-determined likeness threshold compared with post-operative songs. The accuracy score quantifies the magnitude of song fidelity specifically between sound elements that meet the similarly threshold by measuring the local, fine grained similarity (Tchernichovski, et al., 2000). Both scores were used to determine whether different algorithms used to measure song stability were each associated with the number of new neurons.

Bromodeoxyuridine (BrdU) Injections and Tracheosyringeal Denervation

All birds received intramuscular injections of 5-Bromo-2’-deoxyuridine (BrdU; 10 mg/ml in tris buffered saline (TBS), pH 7.40; Sigma) 3x/day for three days to label mitotically active cells. Three weeks after the last injection, animals underwent left (n = 17), right (n = 15) nXIIts nerve resection or sham (n = 17) surgery. 18 - 22 days after the last BrdU injection, birds were anesthetized with either a mixture of ketamine and xylazine (0.03-0.05 mg/g and 0.06 mg/g weight of bird, respectively), or isoflurane in compressed air (3%; Henry Schein). Using a surgical microscope (Zeiss Universal S3B, West Germany), a ~3-mm rostro-caudal incision was made < 1 mm lateral to the ventral midline to expose the trachea, and the tracheosyringeal nerve was isolated from the trachea. A 2 - 4 mm section of either the left or right tracheosyringeal nerve was resected to prevent regrowth following surgery. Control birds received sham surgery in which the same surgery was performed but without section of the tracheosyringeal nerve. Birds were transferred to their preoperative cages once effects from the anesthesia wore off.
Histology

7 - 9 d after surgery birds were overdosed with sodium pentobarbitol (Euthasol, Virbac USA, Fort Worth, Texas) and perfused transcardially with 0.1 M phosphate buffered saline (PBS, pH 7.40; ≤40°F) followed by 4% paraformaldehyde (Sigma-Aldrich; pH 7.40; ≤40°F). The brains were post-fixed for 1 h in 4% paraformaldehyde, rinsed in PBS for 3 h (changing solution hourly), dehydrated in increasing concentrations of ethanol, and embedded in polyethylene glycol (MW = 1500; Polysciences, Warrington, PA). Six-μm sagittal sections were cut on a rotary microtome. Emphasis was put on orienting the brain with the midline parallel to the blade edge. The first complete section through the telencephalon was saved and subsequently every sixth section of tissue was mounted onto Superfrost Plus + slides (VWR Micro Slides). Sections were mounted so that hemisphere could not be identified.

Doublecortin Immunohistochemistry

Doublecortin (DCX) is a protein expressed in migrating and newly established neurons (~1 - 2 weeks old; Brown et al., 2003). DCX immunostaining was performed using an anti-doublecortin goat polyclonal IgG (Santa Cruz Biotechnology) or anti-doublecortin rabbit polyclonal IgG (Abcam). Sections were brought to room temperature in TBS, followed by a 10-min wash in fresh TBS. Sections were then incubated for 30-min in a 2% H₂O₂ solution (97% TBS, 1% methanol, 0.06%, H₂O₂ in 1.94% water) to eliminate endogenous peroxidases. After three 5-min TBS rinses, sections were either incubated for 3 h in biotinylated horse anti-
goat antibody (Vector Laboratories) or biotinylated sheep anti-rabbit antibody (AbCam) in TBS, rinsed again, and exposed for 1 h to an avidin-biotin complex (Vector Laboratories). Sections were then rinsed in TBS and reacted in a solution of 0.04% 3,3’-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories) until the tissue changed color (approximately 15 - 30 min). Following final TBS rinses, sections were dehydrated in ethanols, delipidized in xylene, and cover slipped with Krystalon (Millipore-Sigma).

BrdU/Hu Immunohistochemistry

Sections were brought to room temperature in phosphate buffer (PB, pH 7.40), then exposed to citrate buffer (pH 5.6 - 6.0) at 90 - 95°C for 10 min, followed by a 5 - min wash in PB (37°C), 3 min in 0.28 % pepsin in 400 ml 0.1 M HCl at 37°C, and three 5 min washes in PB at room temperature. Sections then were blocked with 3 % normal donkey serum (Jackson ImmunoResearch Labs) and 0.5 % Triton X-100 in PB for 1 h at room temperature, followed by a 1 - 2 day exposure to sheep anti-BrdU in blocking buffer at 4°C (1:239, Capralogics). Sections were again rinsed in PB and then incubated overnight in biotin-conjugated donkey anti-sheep IgG in PB (1:200, Vector Laboratories), followed by overnight incubation in streptavidin conjugated to Alexa 488 in PB (1:800, ThermoFisher Scientific) for visualization of BrdU. The next day, sections were washed with PB and blocked for one hour with 3% blocking buffer, followed by overnight exposure to mouse anti-Hu primary antibody at 4°C (1:200 in 3% block; Invitrogen). After three 5 min PB rinses at room temperature in the dark, tissue was exposed to donkey anti-mouse IgG conjugated to Cy-3 in PB (1:80; EMD Millipore) for 1 h for visualization of Hu. Sections were then washed in PB, dehydrated with ethanols, delipidized with xylene, and cover slipped with Krystalon (Millipore-Sigma).
Microscopy

Data were collected without knowledge of bird identity, condition, or hemisphere. Area measurements and cell counts for all regions were performed using a computer-yoked light microscope and mapping software (Olympus BX51; Lucivid LED microprojection, Neurolucida, MicroBrightField). The boundaries of all brain regions were traced in ≥6 sections per hemisphere per bird. DCX+ cells were visualized with bright field light microscopy (Figure 12). BrdU+/Hu+ cells were visualized using fluorescein isothiocyanate (FITC) and rhodamine filters and a dual FITC/rhodamine filter (Figure 13). New neurons per square millimeter (nn/mm²) was calculated by dividing the numbers of labeled cells by the total area sampled.

Mapping regions

The location of HVC was identified by the location of the hippocampus, cerebellum, and the robust nucleus of the arcopallium (RA) under dark field. Area X is found below and anterior to the bright oval shaped lateral magnocellular nucleus of anterior nidopallium (IMAN) which is demarcated by a dense haze of terminals and small cells; Area X and IMAN are separated by lamina pallio-subpallialis (LPS). Area X was demarcated by darker boundaries under dark field optics. The boundaries of NCM were determined by both caudal and ventral edges of the brain (Mello et al., 1994). In medial sections, the caudal medial mesopallium (CMM) is adjacent to NCM and separated by a visible lamina. The Field L Complex subdivision L2 is identified under dark field and was seen as a bright band of densely packed cells rich in neuropil (Fortune and Margoliash, 1992; as in Pytte et al., 2010); it is located 300 um rostral to the rostral border of NCM in more lateral sections.
Statistical Analysis

Data are presented as means and SEMs unless otherwise specified. Analyses were performed using one-way ANOVAs, Two-Factor ANOVAs with repeated measures on hemisphere, and Kruskal-Wallis non-parametric tests. Tukey’s post hoc tests were used following significant ANOVAs. Linear regression was used to assess interhemispheric correlations. For all tests, the criterion for significance was set at $p < 0.05$. The Lateralization Index (L.I.) represents the relative number of new neurons between hemispheres, normalized for the bird’s mean number of new neurons in both hemispheres. The formula is arranged such that higher positive values indicate more new neurons in the left hemisphere relative to the right following Tsoi et al., 2014. The formula is as follows:

\[
\frac{\text{Left neurons/mm}^2 - \text{Right neurons/mm}^2}{(\text{L neurons} + \text{R neurons}) / (\text{L area sampled} + \text{R area sampled})}
\]

Results

Song Analysis

We first confirmed that unilateral tracheosyringeal denervation degraded song structure, and compared effects of left and right nXIIIts cuts. Birds that underwent a unilateral nXIIIts-cut had significantly lower accuracy scores compared with controls (One-way ANOVA with three samples, $F = 13.95, p < 0.05$, Figure 14). Post-hoc analyses demonstrated that there were significant differences in accuracy score in each of left nXIIIts-cut and right nXIIIts-cut conditions compared with controls (Tukey’s Post-Hoc, $p < 0.01$) and no difference between the two denervated groups (Tukey’s Post-Hoc, $p > 0.05$). However, similarity scores did not differ
significantly between either left nXIIIts-cut or right nXIIIts-cut birds compared to controls (One way ANOVA with three samples, $F = 1.84, p > 0.05$, Figure 15), which may be due to the greater variability in denervated groups relative to the control group.

*Effects of nXIIIts-cut on Neurogenesis in HVC*

We examined the effect of unilateral nXIIIts-cut on the number of new neurons in HVC that survived to 27 - 30 days of age. The neurons labeled by BrdU/Hu were 18 - 22 days old at the time of syringeal denervation, and were exposed to altered sensory feedback for 7 - 9 days before sacrifice. We found no effect of treatment ($F = 0.14, p > 0.05$), hemisphere ($F = 0.17, p > 0.05$), or treatment by hemisphere interaction on numbers of BrdU/Hu labeled cells (Two-Factor ANOVA with repeated measures on hemisphere, $F = 0.29, p > 0.05$, Figure 16). We then investigated differences in the lateralization of new neurons between the three treatment groups and found that unilateral nXIIIts-cut did not significantly affect the relative number of BrdU/Hu labeled cells in the right hemisphere as compared to the left hemisphere (One-Way ANOVA with three samples, $F = 0.98, p > 0.05$, Kruskal-Wallis, $H(2) = 2.32, p > 0.05$, Figure 17).

Next, we sought to examine whether unilateral nXIIIts-cut affected the number of DCX-labeled neurons (i.e., 1 - 2 week old neurons) in HVC. There was no effect of treatment ($F = 0.48, p > 0.05$), hemisphere ($F = 1.22, p > 0.05$), or treatment by hemisphere interaction on numbers of DCX+ labeled cells ($F = 0.05, p > 0.05$, Two-Factor ANOVA with repeated measures on hemisphere, Figure 18). These data indicate that alteration of sensory feedback by nXIIIts cut did not affect numbers of new neurons in HVC regardless of the age of the neurons.
Effect of nXIIts-cut on Neurogenesis in Area X

We found an effect of treatment (F = 7.47, p = 0.001), but not an effect of hemisphere (F = 0.25, p > 0.05), or an interaction of treatment by hemisphere on numbers of 27 - 30 day old (BrdU/Hu labeled) neurons in Area X (Two-Factor ANOVA with repeated measures on hemisphere, F = 1.16, p > 0.05, Figure 19). Because there was no difference between left and right neuron counts within any of the groups, we combined numbers of BrdU/Hu labeled neurons in the left and right hemispheres in each of the three treatment groups. There was a significant difference in the total number of neurons in Area X among the three treatment groups (One-Way ANOVA with repeated measures on hemisphere, F = 6.99, p = 0.002, Figure 20). Post-hoc analyses demonstrated that there were significant differences in numbers of 27 - 30 day old neurons between the control and left nXIIts-cut group (Tukey’s Post-Hoc, p < 0.01) and between control and the right nXIIts-cut group (Tukey’s Post-Hoc, p < 0.05). There were no significant differences in the number of 27 - 30 day old neurons between the two denervated groups (Tukey’s Post-Hoc, p > 0.05). These data demonstrate that the survival of adult born neurons in Area X is affected by sensory feedback. We also found that unilateral nXIIts-cut did not affect the relative numbers of new neurons in the right and left hemispheres (One-Way ANOVA with three samples, F = 0.2, p > 0.05, Figure 21).

Decreased numbers of new neurons in Area X in the denervated groups may be due to a disruption in new neuron recruitment to Area X or decreased survival of new neurons after reaching Area X. To differentiate between these, we examined DCX, expressed in younger (1 -2 week old) neurons. There was no effect of treatment (F = 0.26, p > 0.05), hemisphere (F = 0.68, p >.05), or treatment by hemisphere interaction on the number of 1 - 2 week old neurons in Area X (Two-Factor ANOVA with repeated measures on hemisphere, F = 0.36, p > 0.05, Figure 22).
Unilateral nXIIIts-cut also did not affect the relative numbers of young neurons between hemispheres (One-Way ANOVA with three samples, F = 0.18, p > 0.05, Kruskal-Wallis, H(2) = 0.47, p > 0.05, Figure 23). Given this, we concluded that the observed decrease in 27 - 30 day old neurons in Area X was not due to a perturbation in the recruitment of immature neurons. Instead, it is more likely due to a change in the local microenvironment that adversely affected new neuron survival.

Effect of nXIIIts-cut on Neurogenesis in NCM

Finally, we examined the effect of unilateral nXIIIts-cut on the number of 27 - 30 day old neurons in NCM. We found no effect of treatment (F = 1.36, p > 0.05), hemisphere (F = 1.41, p > 0.05), or treatment by hemisphere interaction on numbers of BrdU+/HU+ cells in NCM (Two-Factor ANOVA with repeated measures on hemisphere, F = 1.96, p > 0.05, Figure 24). However, when we investigated lateralization differences between the three treatment groups, we found an overall trend towards a significant difference in the lateralization index among the groups (One-Way ANOVA with three samples, F = 3.24, p = 0.055, Figure 25), such that there was greater right lateralization of new neurons in the right nXIIIts-cut group compared with the control group.

There was no effect of treatment (F = 1.19, p > 0.05), hemisphere (F = 0.08, p > 0.05), or treatment by hemisphere interaction on numbers of DCX+ 1 - 2 week old neurons (F = 1.22, p > 0.05, Two-Factor ANOVA with repeated measures on hemisphere, Figure 26). We found an overall difference in the lateralization index of DCX+ neurons among the groups (One-Way ANOVA with three samples, F = 4.51, p = 0.04, Figure 27). Post-hoc analyses showed that there were significant differences in 1 – 2 week old neurons in the control versus right nXIIIts-cut group (Tukey’s Post Hoc, p < 0.05), but no differences between the control and left nXIIIts-cut
group (Tukey’s Post-Hoc, p > 0.05) or between denervated groups (Tukey’s Post-Hoc, p > 0.05). These findings demonstrate that there is greater right lateralization of neurons in the control group versus the right nXIIts-cut group during neuronal recruitment, however, there is a trend towards reversal of this pattern at the level of neuronal survival.

Correlations in new neurons between hemispheres within regions
We also determined whether the number of 27 - 30 day old new neurons in HVC, Area X, or NCM co-varied across hemispheres within individuals. This provides an indication of whether new neuron survival is similarly regulated cross-hemisphere within a given region, or whether each brain region is independently modulated within the hemisphere.

We did not find a correlation between the numbers of new neurons in left and right HVC of the control group (r = 0.19, p > 0.05) or left nXIIts-cut group (r = 0.11, p > 0.05). However, there was a positive correlation between number of new neurons in left and right HVC of the right nXIIts-cut group (r = 0.47, p = 0.02; Figure 28). The removal of the highest data point resulted in a loss of the correlation (r = 0.23, p > 0.05). We did not find correlations between left and right hemispheres of Area X in the control group (r = 0.06; p > 0.05), left nXIIts-cut group (r = .03, p > 0.05) or the right nXIIts-cut group (r = 0.18, p > 0.05, Figure 29). Similarly, there was no correlation between left and right NCM in the control group (r = 0.25, p > 0.05), left nXIIts-cut group (r = 0.13, p > 0.05), and right nXIIts-cut group (r = 0, p > 0.05, Figure 30).

We also measured whether the number of 1 – 2 week old neurons in HVC, Area X, or NCM co-varied across hemispheres within individuals. We did not find correlations between left and right hemispheres of HVC in the control group (r = 0.07, p > 0.05) or left nXIIts-cut group (r = 0.32, p > 0.05), but there was a trend towards a significant correlation in the right nXIIts-cut
group \((r = 0.60, p = 0.07, \text{Figure 31})\). Removal of the highest data point resulted in a loss of this trend \((r = 0.58, p > 0.05)\). There was a significant correlation between the numbers of new neurons in left and right hemispheres of Area X in the control group \((r = 0.84, p = 0.00)\) and left NXIIIts-cut group \((r = 0.64, p = 0.01)\), but not in the right NXIIIts-cut group \((r = 0.28, p > 0.05, \text{Figure 32})\). We did not find a correlation between the numbers of new neurons in left and right NCM of the control group \((r = 0.23, p > 0.05)\), left NXIIIts-cut group \((r = 0.20, p > 0.05)\), or right NXIIIts-cut group \((r = 0.30, p > 0.05, \text{Figure 33})\).

**Table 2. Correlations in 27 – 30 day old neurons between brain regions within hemispheres**

<table>
<thead>
<tr>
<th>Group</th>
<th>CONTROL</th>
<th>LEFT NXIIIts-cut</th>
<th>RIGHT NXIIIts-cut</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hemisphere</td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td></td>
<td>HVC – Area X</td>
<td>(r = 0.49,) (p = 0.19)</td>
<td>(r = 0.01,) (p = 0.90)</td>
</tr>
<tr>
<td></td>
<td>HVC – NCM</td>
<td>(r = 0.83,) (p = 0.09)</td>
<td>(r = 0.01,) (p = 0.86)</td>
</tr>
<tr>
<td></td>
<td>Area X – NCM</td>
<td>(r = 0.60,) (p = 0.09)</td>
<td>(r = 0.60,) (p = 0.09)</td>
</tr>
</tbody>
</table>

Bonferroni corrected values show that 27 – 30 day old neurons were not correlated across brain regions in any of the treatment groups \((p > 0.05\) for all).

**Table 3. Correlations in 1 – 2 week old neurons between brain regions within hemispheres**

<table>
<thead>
<tr>
<th>Group</th>
<th>CONTROL</th>
<th>LEFT NXIIIts-cut</th>
<th>RIGHT NXIIIts-cut</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hemisphere</td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td></td>
<td>HVC – Area X</td>
<td>(r = 0.40,) (p = 0.25)</td>
<td>(r = 0.37,) (p = 0.27)</td>
</tr>
<tr>
<td></td>
<td>HVC – NCM</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Area X – NCM</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
Bonferroni corrected values show that 1 – 2 week old neurons were not correlated across brain regions in any of the treatment groups (p > 0.05 for all). Correlations in 1 – 2 week old neurons between HVC-NCM and Area X-NCM were unable to be obtained in the control condition due to low sample sizes.

**Discussion**

We found that irreversible disruption to song structure by unilateral denervation of the syrinx in adult male zebra finches impacted numbers of 27 – 30 day old neurons in a region and hemisphere-specific manner. Interestingly, existing models of new neuron survival predict that aberrant song feedback may either increase or decrease new neurons. An increase in new neuron survival has been suggested to occur in response to an increased “demand” or activity level following mismatched feedback, as incoming neurons may function in attempting to correct the song, or to “rewrite” the bird’s representation of his new song (Wilbrecht et al., 2002a; Wilbrecht et al., 2002b; Pytte et al., 2011). It has also been suggested that a decrease in new neuron survival may occur in response to poor song quality as neurons that contribute to error signals are culled from the population (Wilbrecht and Kirn, 2004; Pytte et al., 2011). We found that altering song production via NXIIIts-nerve cuts resulted in decreased 27 – 30 day new neuron survival in Area X, loss of left-sided lateralization of neurons in NCM, and had no effect in HVC. This finding indicates that the effects of syringeal denervation on neurogenesis vary widely throughout the brain, depending both on the region and hemisphere that receive new neurons.

In regions that receive new neurons in adulthood, roughly half or more of the young neurons that are formed are naturally culled within the first month after cell birth-dating (Gould et al., 1999; Kirn et al., 1999). In both rodents and songbirds, numerous experiences during this culling period “rescue” young neurons that would otherwise die, including wheel running (van
Praag et al., 1999; Marlaat et al., 2012), learning (Gould et al., 1999; Curlik and Shors, 2011), environmental enrichment (Kempermann et al., 1997) and activity of that particular brain region (Li et al., 2000; Pytte et al., 2010). For example, in adult zebra finches, increased singing rate is positively correlated with numbers of new neurons in HVC, which houses the motor pattern for song production (Li et al., 2000), and deafening decreases numbers of new neurons in NCM, an auditory region specific for song processing (Pytte et al., 2010), indicating that new neuron survival in song regions underlying song behavior is influenced by that behavior. Interestingly, the effects of singing behavior or song structure on new neurons in Area X have not previously been investigated.

*Altered auditory/sensorimotor feedback does not impact new neurons in HVC*

Pytte et al. (2011) demonstrated that the degree of song recovery following botox-induced paralysis of syringeal vocal muscles was positively correlated with increased survival of new neurons in HVC of adult male zebra finches. However, botox paralysis is temporary, and thus the song structure was temporarily, rather than permanently, degraded. Therefore, it was unclear whether song recovery increased the survival of new neurons in HVC, or if increased neuronal survival improved song recovery. The present study attempted to establish the directionality of these results by denervating one half of the syrinx to permanently alter song structure and assess whether there was an effect on new neurons in HVC. We found no effect of altered song quality on new neuron survival in HVC, suggesting that in Pytte’s work (2011), new neuron survival drove recovery of song structure, and not vice versa.

Wilbrecht et al (2002a) demonstrated that juvenile zebra finches that underwent unilateral syringeal denervation early in the song learning process transiently recruited twice the number of
new neurons to the side of HVC with an intact syringeal nerve (Wilbrecht et al., 2002a). It is important to note that due to their early developmental stage, song stereotypy had not yet been achieved in these birds. Therefore, it is possible that survival of new neuron density increased in HVC on the side of the intact syringeal nerve due to an increase in “demand,” and that placing such a demand allowed song learning with only one side of the syrinx. The difference in developmental stages between the juvenile birds in Wilbrecht et al. (2002a) and the adult birds in our current study may explain the difference in results. Overall, it appears that numbers of new neurons in HVC in adult male zebra finches may be less susceptible to disruption by altered sensory feedback.

To determine whether altered sensory feedback disrupts normal synaptic activity in HVC, Vallentin & Long (2015) unilaterally denervated the syrinx of adult male zebra finches and measured intracellular electrical activity in HVC projection neurons (i.e., those projecting from HVC to RA and HVC to Area X). In line with our findings, Vallentin & Long (2015) found that synaptic activity of HVC projection neurons were not affected by unilateral denervation, suggesting that synaptic activity in HVC of adult zebra finches is independent of sensory feedback. Interestingly, several other studies have also suggested that HVC adult neuron number is independent of sensory experience. For instance, the total number of HVC neurons in zebra finches deafened early in development was similar to the number of neurons seen in intact controls (Hurley et al., 2008). Brenowitz et al. (1995) also demonstrated that marsh wrens that were taught large song repertoires had no more HVC neurons than those that were taught smaller repertoires. New neurons in HVC project to RA (Alvarez-Buylla et al., 1990; Kirn et al., 1991) and have been shown to respond to auditory stimuli (Paton and Nottebohm, 1984). However, there is currently a lack of evidence suggesting that neurons in HVC are responsive to the bird’s
own song during singing (Leonardo, 2004; Kozhevnikov and Fee, 2007; Bauer et al., 2008). This lack of auditory responsiveness during singing raises the question of whether auditory sensitivity is gated during singing such that self-generated auditory feedback does not reach HVC (Schmidt and Konishi, 1998; Cardin and Schmidt, 2004). If so, the mechanism underlying gating is unclear. However, there is evidence to suggest that it may occur at the level of HVC through either cholinergic modulation from the forebrain (Shea and Margoliash, 2003) or through direct influences from thalamic nucleus uvaeformis, a region that is critical for normal song production (Akutagawa and Konishi, 2005; Coleman et al., 2007). The gating of auditory responses is likely controlled at the level of HVC because auditory responses are not suppressed in the upstream region of field L, which is one of the primary auditory inputs to HVC. However, others have suggested that this process does not occur at the level of HVC, but rather at the level of the nucleus interfacialis of the nidopallium, (NIf), a nucleus that sits at the interface of the auditory and song systems (Lewandowski et al., 2013). Regardless of the anatomical origin of this process, gating represents a mechanism for filtering out unnecessary auditory stimuli while allowing the processing of behaviorally relevant auditory signals (Schmidt & Konishi, 1998). This unresponsiveness of auditory stimuli in HVC may provide an explanation for the lack of effect of altered singing on new neuron numbers.

Finally, we cannot rule out the possibility that altered auditory feedback did not impact new neuron numbers in HVC due to the timeline of our experiment. Newborn neurons have critical periods during which their survival is sensitive to different environmental stimuli (Yamaguchi and Mori, 2005; Magavi et al., 2005). Alvarez-Buylla (2004) demonstrated that administration of BDNF into HVC 14-20 days after new neurons are born grants them a longer life expectancy. However, this extended life expectancy does not occur when BDNF is infused in
HVC 10 days earlier or later. This suggests that the life expectancy of HVC neurons is determined by BDNF during a critical period soon after these neurons reach the destination of HVC. Similarly, our study demonstrates that there is no effect on the number of new neurons when auditory feedback is altered through NXIIIts-cuts 21 days after the birth of neurons. However, neurons reach HVC from their birth site (i.e., ventricular zone of the lateral ventricles) within 7 days, and thus, it is possible that if NXIIIts-cuts were made at an earlier time point, we would be able to capture the impact of altered auditory feedback on new neuron survival. Such a finding is certainly within the realm of possibilities considering that previous research demonstrates that new neuron survival is influenced by the environment during a narrow temporal window.

*Altered auditory feedback affects numbers of new neurons in Area X*

There are two types of song changes that occur after unilateral denervation of the syrinx: immediate changes in song acoustic structure due to loss of control over half of the syrinx, and changes in the central motor pattern that appear after several weeks. The mechanism underlying the central change is unclear (William and McKibben, 1992); however, it is known that zebra finches continue to use auditory feedback to monitor their song throughout adulthood (Nordeen and Nordeen 1992; Lombardino and Nottebohm, 2000). The anterior forebrain pathway (AFP) has been shown to produce changes to the song motor pathway in response to vocal errors and it has been suggested that the AFP plays a role in monitoring song feedback. We propose that this monitoring function is also influencing neuron survival in Area X after NXIIIts-nerve cuts.

We found fewer new neurons in Area X of birds who were unable to produce their preoperative song. It seems that the effects of the treatment were not specific to either an
ipsilateral or contralateral effect of the nerve cut itself. Thus, either a unilateral nerve cut retrogradely affected both hemispheres equally through aberrant proprioceptive feedback, or the effect was due to a bilateral outcome of the treatment, such as altered auditory feedback.

It is unlikely that nerve section causes song degradation via aberrant proprioceptive feedback for several reasons. First, removal of the afferent fibers in the tracheosyringeal nerve does not trigger decrystallization of adult zebra finch song, which suggests that proprioceptive input from the syrinx alone is not necessary for song maintenance (Bottjer et al., 1984). In addition, anatomical studies suggest that the forebrain nucleus interface of the nidopallium (NIf) is a major conduit through which proprioceptive information might reach HVC. However, if this information were in fact necessary for song maintenance, NIf lesions would be expected to trigger decrystallization of song, but such findings have not been observed (Cardin et al., 2005). In addition, there are no known bilateral projections from the nXIIIts into the song system, nor bilateral downstream projections that could be retrogradely degenerated. Finally, song degradation as a result of nerve section, and other forms of degradation induced by manipulations of auditory signals (i.e., deafening), require an intact AFP and act over a slow time course, which suggests that similar central pathways are involved in these processes. Thus, it is likely that the main mechanism by which the tracheosynringeal nerve section is inducing a decrystallization of song is through altered auditory feedback.

It was initially proposed that song production occurred through a learned central pattern generator and adult birds no longer monitored either auditory or proprioceptive feedback during song production (Konishi, 1965, 1969). This long-standing model was overturned when it was demonstrated by deafening adult zebra finches that auditory feedback during singing was necessary for song maintenance (Nordeen and Nordeen, 1992). The result of deafening was
gradual song degradation over the course of weeks. Interestingly, bilateral lesions of IMAN, the output pathway of the AFP, prevented song changes that otherwise occur after deafening, suggesting a role for the AFP in inducing song changes in the motor pattern following altered song feedback. The AFP plays a critical role in evaluating vocal errors and translating changes in auditory feedback to changes in song output. When feedback is disrupted, such as in the case of mismatched song to an internal template of the bird’s song, the AFP may generate an error-reducing bias, which could increase vocal exploration and instruct synaptic changes in the motor pathway. In the present study, the feedback, which is poorly matched to the representation of the tutor’s song or the bird’s own song, may in turn lead to decreased survival of new neurons in Area X.

*Altered auditory/sensorimotor feedback alters left-side lateralization of new neurons in NCM*

In NCM, numbers of new neurons in both hemispheres combined were not affected by altered song production; however, there was a trend towards the treatment altering the relative numbers of new neurons across hemispheres. There are normally more new neurons in the left hemisphere NCM than in the right in untreated zebra finches (Tsoi et al., 2014). The NxIIIts-cuts decreased the degree of left hemisphere lateralization compared to controls. This led us to posit that left and right NCM may contribute differently to processing self-song information, and thereby the effect of altered feedback on neuron survival was similarly lateralized.

The degree of left side lateralization of new neurons is correlated with the quality of song imitation and the strength of neuronal memories for conspecific songs (Tsoi et al., 2014). The findings by Tsoi et al. (2014) reflect immediate early gene (e.g. *zenk*) expression in response to playback of the tutor’s song. Left lateralized *zenk* expression in NCM only occurred when
juveniles heard playback of their father’s song but not when they heard unfamiliar conspecific songs or silence. Thus, it is possible that left side NCM holds a stronger memory of the tutor song than does right NCM (Moorman et al., 2012). Other work supports the idea that the left hemisphere of NCM plays a role in the memory of the tutor’s song, as well as the bird’s own song (Bolhuis and Gahr, 2006; Phan et al., 2006; Canopoli, 2014).

Taken together, it is possible that a mismatch of the bird’s song with the representation of either the tutor’s song or the bird’s own song causes a decrease in survival of new neurons in left NCM, as the left NCM appears to play a larger role in the memory of both internal song templates.

Figure 11. Diagram of the song system. Red lines represent the anterior forebrain pathway which is required for song learning and song maintenance. Blue lines represent the vocal/song
motor pathway which is required for song production. HVC (proper name), Area X, and NCM receive new neurons throughout adulthood (reproduced from Mooney et al., 2004).

**Figure 12.** Photomicrograph of a doublecortin-positive neuron in HVC labeled with DAB (60x). Scale bar, 25 µm.

**Figure 13.** Photomicrographs of the same view (60x) of HVC showing fluorescent markers used to identify newly formed neurons. From left to right, arrows show BrdU-labeled nucleus (FITC filter), Hu-labeled cytoplasm (rhodamine filter), and co-localization of the markers (rhodamine/FITC filter). Scale bars, 10 µm.

![Image](image_url)
Figure 14. There were significant differences in song accuracy scores across the three treatment groups. Mean accuracy scores were significantly different between the control condition and left nXIIIts-cut condition, and the control condition and right nXIIIts-cut condition. There were no differences between mean accuracy scores between denervated groups.
**Figure 15.** Mean percentage similarity scores were not significantly different across treatment groups.

**Figure 16.** There were no differences in numbers of 27-30 day old neurons in HVC among NXIIIts-cut and control birds.
Figure 17. There were no differences in the lateralization index of 27 - 30 day new neurons in HVC among nXIIIts-cut and control birds.
**Figure 18.** There were no differences in the number of 1 - 2 week old neurons in HVC among NXIIIts-cut and control birds.

**Figure 19.** NXIIIts-cut birds had fewer 27 - 30 day old neurons bilaterally in Area X compared to controls.
Figure 20. The combination of hemispheres in Area X across groups also resulted in significantly fewer 27 - 30 day old neurons in denervated groups.

![Graph showing lateralization index]

Figure 21. There were no differences in the lateralization index of 27 - 30 day new neurons in Area X among nXIIIts-cut and control birds.

![Graph showing 1-2 week old neurons/mm²]
Figure 22. There were no differences in the number of 1 - 2 week old neurons in Area X among nXIIts-cut and control birds.

Figure 23. There were no differences in the lateralization index of 1 – 2 week old neurons in Area X among nXIIts-cut and control birds.
Figure 24. There were no differences in the number of 27 - 30 day old neurons in NCM among nXIIts-cut and control birds.

Figure 25. There was a trend towards a significant difference in the lateralization index of 27 – 30 day old neurons among the three treatment groups in NCM.
Figure 26. There were no differences in the number of 1 – 2 week old neurons in NCM among NXIIIts-cut and control birds.

Figure 27. There were lateralization differences between the three treatment groups in NCM. There were significantly greater number of 1 – 2 week old neurons entering the right hemisphere.
than the left hemisphere in the control group versus the right NXIIIts-cut condition. There were no lateralization differences between the control and left NXIIIts-cut condition or between both denervated groups.

![Graph showing the correlation between 27-30 day old neurons (mm²) in left HVC and right HVC. The correlation coefficient is r = 0.19 and p = 0.10.]
Figure 28. There was no correlation between numbers of 27 – 30 day old neurons in left and right HVC in the control group (A) or after a left NXIIIts-cut (B) but there was a correlation in the right NXIIIts-cut group (C).
27-30 day old neurons (mm$^2$) in left Area X

27-30 day old neurons (mm$^2$) in right Area X

$r = 0.06$
$p = 0.35$

Area X - Control

B

27-30 day old neurons (mm$^2$) in left Area X

27-30 day old neurons (mm$^2$) in right Area X

$\text{r} = 0.03$
$p = 0.54$

Area X - Left NXIIts-cut
Figure 29. There was no correlation between numbers of 27 – 30 old neurons in left and right Area X in the control group (A) or after a left NXIIIts -cut (B) or right NXIIIts -cut (C).
Figure 30. There was no correlation between numbers of 27 – 30 day old neurons in left and right NCM in the control group (A) or after a left NXIIIts-cut (B) or right NXIIIts-cut (C).
A. HVC- Control

![Graph showing a positive correlation between 1-2 week old neurons in left HVC and right HVC. The correlation coefficient is r = 0.07 and p = 0.62.]

B. HVC- Left NXIIIts-cut

![Graph showing a positive correlation between 1-2 week old neurons in left HVC and right HVC. The correlation coefficient is r = 0.32 and p = 0.19.]

96
Figure 31. There was no correlation between numbers of 1 – 2 week old neurons in left and right HVC in the control (A), left NXIIIts-cut (B), or right NXIIIts-cut group (C).
1 - 2 week old neurons (mm$^2$) in left Area X

r = 0.84
p = 0.00

1 - 2 week old neurons (mm$^2$) in right Area X

1 - 2 week old neurons (mm$^2$) in left Area X

r = 0.64
p = 0.01

Area X - Control

Area X - Left NXIIIts-cut

1 - 2 week old neurons (mm$^2$) in right Area X
Figure 32. There was a significant positive correlation between numbers of 1–2 week old neurons in left and right Area X in the control (A) and left NXIIIts-cut (B) groups, but not in the right NXIIIts-cut group (C).
Figure 3.3. There was no correlation between numbers of 1 – 2 week old neurons in left and right NCM in the control (A), left NXIIIts-cut (B), or right NXIIIts-cut group (C).
CHAPTER 4: GENERAL DISCUSSION

Adult neurogenesis has been implicated in cognitive functioning, and alterations in this process have been associated with changes in cognition, particularly in the areas of learning and memory. Neurodegenerative disorders and brain injury have been known to alter neurogenesis. However, whether new neurons are uniformly altered across different brain regions and across hemispheres within the same brain regions had not been explored until now. One of the present studies used a transgenic Huntington’s disease rat model to examine whether the relationship between neurogenesis and quality of behaviors was similarly altered across brain regions and hemispheres during a neurodegenerative process. We used a songbird model to further explore whether altered sensory feedback, which is a proposed mechanism by which motor dysfunction occurs in HD, alters neurogenesis similarly across and within different brain regions. The goal was to provide an understanding of whether neurogenesis is similarly altered across brain regions and hemispheres that are impacted after neurodegeneration and/or injury. Such an understanding would provide insight into appropriate treatment options based on the impacted brain region and/or hemisphere. The ultimate goal is for this information to assist in the reversal of cognitive impairments that are associated with neuronal loss after a neurodegenerative process or injury.

In my first study I compared the recruitment of new neurons in the hippocampus and SVZ between a transgenic Huntington’s disease rat model (tgHD) and wild-type (WT) rats. I also compared the relationship between new neurons in the hippocampus and SVZ with performance on a timing task, which is a behavior that is commonly affected throughout this disease process, and may account for HD symptoms (i.e., motor impairments, slow processing speed, attention, memory). This study is the first to demonstrate that the relationship between adult neurogenesis and timing behavior is altered in the hippocampus of a tgHD rat model.
Interestingly, greater numbers of new neurons in the hippocampus and SVZ of WT rats were associated with worse performance on a timing task, whereas greater number of new neurons in the hippocampus of tgHD rats were associated with improved performance on a timing task. There were no differences between genotypes in the numbers of new neurons in the hippocampus or SVZ within each hemisphere. Likewise, there was also no difference between genotypes in numbers of new neurons within a brain region between the left and right hemispheres. Rather, differences were observed between genotypes in the relationship between new neurons and a timing behavior. Given that HD predominantly affects the striatum, we expected that tgHD rats would have a greater number of new neurons in the SVZ than the SGZ to compensate for the loss of neurons in the striatum. This lack of a difference in the total number of new neurons in the SGZ and SVZ between genotypes possibly reflects the secondary effects that occur in response to neurodegeneration, which impacts neurogenesis at a global level, and thus has similar effects on new neurons across brain regions.

Endogenous factors that influence neurogenesis, such as cytokines, chemokines, neurotransmitters, and reactive oxygen/nitrogen species, are released by dying neurons and activated macrophages, microglia, and astrocytes during neurodegeneration (Yoneyama et al., 2011). Evidence of the accumulation of reactive microglia and astrocytes has been observed in brains from patients with HD (Yang et al., 2017; Sapp et al., 2001). PET imaging showed that microglia activation correlates with the pathology in patients with HD, and these cells accumulate in relation to the degree of neuronal loss in the striatum, hippocampus, and cortex. In addition to the accumulation of microglia, dying neurons are found in the striatum and hippocampus of HD patients and animal models (Spargo et al., 1993; Jakel & Maragos, 2000). Dying neurons then activate endogenous factors that are likely contributing to the regulation of
new neurons in both of these regions, and may explain why there were no differences in the number of new neurons in the SVZ and SGZ of tgHD rats.

Given that endogenous factors released during neurodegeneration increase neurogenesis, it is interesting that in the present study there were no differences in new neuron numbers between tgHD rats and WT rats. One of the proposed reasons for the lack of difference in new neuron numbers between genotypes is the age of the rats at the time in which neurogenesis was measured. In both rodents and humans, the rate of decline of new neurons increases with age (Seki and Arai 1995; Kuhn et al 1996; Kempermann et al 2002; Gould, et al., 1999), with peaks in age-related decreases occurring between 3 and 12 months in rats (Kuhn et al., 1996). The rats in our present study were either 21 or 24 months, and thus it is likely that they had already undergone age-related declines in neurogenesis that resulted in similar new neuron density across tgHD and WT rats. Perhaps differences in new neuron numbers across genotypes would be observed if neurogenesis in rats were observed across varying time points throughout their life span.

One of the most detrimental symptoms of Huntington’s disease includes motor dysfunction known as chorea: rapid, arrhythmic and complex involuntary movements. Chorea appears to be an effect of dysfunctional error-feedback processing, or the processing of errors and performance monitoring (Smith et al., 2000; Beste et al., 2006). One of the ways in which error-related processes is assessed in humans is via event-related potentials (ERP), called error negativity (Ne). Ne is a component of the ERP and has been associated with acknowledged incorrect responses that occur in discrimination tasks (Falkenstein et al., 1991; Gehring et al., 1993; Dehaene et al., 1994). The Ne is related to the execution of an incorrect response, is absent for trials on which the correct response is made when the subject is certain of the correctness of
that response (Coles et al., 2001), and does not seem to be dependent on the type of error made. The Ne is classically interpreted as the detection by the subject of a mismatch between response representations (i.e., expected response vs. observed response) that is driven by the midbrain dopamine system (DA-system). The DA-system supervises and evaluates events, such as responses. If an event is not as expected, the DA system sends an error signal to the anterior cingulate cortex which in turn elicits the Ne. Whether error feedback processing plays a role in neurogenesis has not been previously explored. Therefore, in an attempt to understand the relationship between new neurons and error feedback processing, we used a songbird model to examine whether new neurons are altered across regions and hemisphere as a result of altered sensory feedback.

I examined whether aberrant sensory feedback via peripheral nerve injury affects the recruitment of new neurons between hemispheres and across three brain regions that underlie singing behavior (i.e., HVC, Area X, and NCM) in a songbird model. To produce aberrant feedback, I unilaterally sectioned the tracheosyringeal nerve which innervates the vocal organ, resulting in altered auditory and sensorimotor feedback during song production. This produced a mismatch between the bird’s motor commands and expected sensory feedback. I discovered that altering song production decreased numbers of new neurons bilaterally in the region of the striatum that functions in monitoring song-related motor behavior (Area X). In the auditory processing region (NCM), I found a trend towards a significant left-sided dominance in number of new neurons in the left hemisphere compared to the right in control birds. Altered song production did not impact new neurons in the song production region (HVC). This work demonstrates that the quality of a behavior differentially impacts neurogenesis in regions that process the behavior.
Regional and hemispheric differences in neurogenesis after mismatched sensory feedback may be due to the distinct role of each nucleus in song behavior. HVC produces stereotyped patterns of neuronal activity that result in production of consistent renditions of learned song. Thus, a lack of susceptibility to aberrant auditory feedback may be adaptive in maintaining song stereotypy. Area X, however, is thought to evaluate ongoing vocal output to assess whether it matches the internally stored template of the bird’s own song, and NCM is involved in auditory perception of learned songs. Therefore, the alterations of new neurons in Area X and NCM after mismatched sensory feedback highlights the importance of these regions in song error-monitoring and perception. Furthermore, the widespread differences in the manner in which new neurons are impacted across HVC, Area X, and NCM demonstrates the diversity of the functional role of each nucleus, and perhaps the diversity of the role of new neurons.

The results of the study illustrate that new neurons are impacted in regions that play a role in the monitoring and perception of error signaling. It is possible that new neurons would be similarly impacted in HD rodent models and patients with HD with chorea given that this symptom appears to be an effect of dysfunctional error-feedback processing (Smith et al., 2000; Beste et al., 2006). Therefore, an examination of new neurons in regions that underlie the evaluation and monitoring of sensorimotor abilities in HD would be worth exploring.

*The Role of Neurogenesis in Neurodegenerative disease*

Evidence for the role of new neurons in neurodegenerative diseases has thus far been inconsistent in the literature. Some studies suggest increases in neurogenesis in diseased/damaged brain regions of human patients, while other studies demonstrate decreases in new neurons in the same brain regions of rodent models. The reasons for such discrepancies
across mammalian brains and brain regions are important to consider. For example, there is extensive literature related to Alzheimer’s disease (AD), which is a neurodegenerative disease with prominent histopathological hallmarks (i.e., amyloid-beta plaque burden). It has typically been found that decreased hippocampal neurogenesis in AD mouse models coincide with amyloid-beta plaque pathology (Lazarov & Marr, 2010; Kuhn et al., 2007). On the other hand, in postmortem Alzheimer’s disease human brains, the number of hippocampal cells expressing immature neuronal markers was elevated in patients with AD pathology compared to age-matched healthy controls (Jin et al., 2004). One hypothesis for this finding in human brains is that increased neurogenesis might be a compensatory mechanism to replace the lost neurons and that stimulating hippocampal neurogenesis might provide a new treatment strategy for AD. In a similar vein, multiple transgenic rodent models have also been generated to replicate the pathology of Huntington’s disease. While a dramatic reduction in hippocampal neurogenesis (Lazic et al., 2004; Gil et al., 2005) has been shown in HD rodent models, no significant differences in SVZ neurogenesis has been found (Gil et al., 2005; Lazic et al., 2006; Moraes et al., 2009). In the present study, no alterations were found in neurogenesis in either the hippocampus or the SVZ of a transgenic HD rat model. On the other hand, studies in patients with HD postmortem tissue revealed an increase in SVZ cell proliferation (Curtis et al., 2003), which may suggest a compensatory mechanism for the significant neuronal loss in the striatum. In the hippocampus, no differences in neurogenesis have been found between patients with HD and healthy controls. The discrepancies described between new neurons in HD rodent models and patients with HD may be due to differences in disease progression and pathology across species.
Sex differences in brain lateralization

Differences in brain structure and function exist between sexes (Allen et al., 2003; Barta & Dazzan, 2003; Chen et al., 2007). In males, brain volume increases are predominantly found in bilateral limbic areas and left posterior cingulate gyrus, whereas higher densities are mostly limited to the left side of the limbic system (Ruigrok et al., 2014). On the contrary, larger brain volume in females is most apparent in areas of the right hemisphere related to language, in addition to several limbic structures such as the right insular cortex and anterior cingulate gyrus (Ruigrok et al., 2014). With regards to functional differences, functional MRI studies have shown that males are more strongly left lateralized during language tasks, whereas females show greater bilateral activity during verbal language tasks (Clements et al., 2006). The stronger left-sided lateralization in response to language tasks in males suggests that they rely less on their right hemisphere for language abilities, which may explain why they have a greater incidence of aphasia after a stroke in the left hemisphere (McGlone, 1977; Inglis and Lawson, 1983).

Despite a growing understanding of the lateralized structural and functional differences between male and female brains, less is known about the lateralized differences in neurogenesis across sexes. Relative to males, female rats and voles have greater proliferation of new neurons in the amygdala, hippocampus, SVZ, and hypothalamus due to the stimulating effects that estradiol (i.e., primary female sex hormone) has on neurogenesis (Tanapat et al., 1999; Smith et al., 2001; Arimatsu and Hatanaka, 1986; Chowen et al., 1992). However, it has yet to be explored whether there are lateralized differences in neurogenesis across sexes. The factors that influence the asymmetric effect that sex has on brain structure, function, and mechanism, may help us understand how and why male and female brains differ in their predisposition for risk to certain diseases and disorders.
Conclusion

Further work is needed to understand the variability in alterations in neurogenesis between neurodegenerative rodent models and human patients with neurodegenerative disorders. The discrepant findings demonstrate that the involvement of new neurons may depend on numerous factors, including the age of neurons (Gu et al., 2012), the age of the animal and/or patient (Martinez et al., 2012), the sex of the animal and/or patient (Chow et al., 2013; Yagi & Galea, 2018), different genotypes of animal models (Wolf et al., 2006), different methods used to assess neurogenesis (Winner & Winkler, 2015), and the different stages or progression of the disease or pathology (Winner & Winkler, 2015). Differences in each of these factors have been found to affect numbers of new neurons (Chow, 2013; Spritzer et al., 2017; Diaz et al., 2009).

In conclusion, results from both of the present studies demonstrate that neurogenesis is regulated across brain regions and within hemispheres in a Huntington’s disease rat model and after sensory feedback is altered in a songbird model. The findings of our study highlight the importance of understanding the relationship between functional behaviors and neurogenesis in healthy brains, as this relationship can change after a disease process begins. On a greater scale, these results provide further evidence for the plastic nature of the brain. Understanding that alterations in neurogenesis, or alterations in the relationship between neurogenesis and the quality of a behavior, is impacted after insult to the brain lends to the possibility of successful treatment options in response to neurodegeneration and/or injury.


André, V. M., Cepeda, C., & Levine, M. S. (2010). Dopamine and glutamate in Huntington’s Disease: A balancing act. CNS Neuroscience &amp; Therapeutics, 16(3), 163-178.


maze learning in two rat strains increases the expression of the polysialylated form of the neural cell adhesion molecule in the dentate gyrus but has no effect on hippocampal neurogenesis. Behavioral Neuroscience, 119(4), 926-932.


Brito, V., & Ginés, S. (2016). P75NTR in Huntington's disease: Beyond the basal ganglia. Oncotarget, 7(1).


Curlik, D. & Shors, T. (2011). Learning increases the survival of newborn neurons provided that learning is difficult to achieve and successful. *Journal of Cognitive Neuroscience*, 23(9), 2159-2170.

deBoo, G., Tibben, A., Lanser, J., Jennekens-Schinkel, A., Hermans, J., Maat-Kievit, A., &
Gene for Huntington Disease. Archives of Neurology, 54(11), 1353-1357.
adult hippocampal neurogenesis affect learning and memory? Nature Reviews
cognitive task: Evidence for a distinction between selection and search. NeuroImage,
7(4), 368-376.
DeWulf, V., & Bottjer, S. (2002). Age and sex differences in mitotic activity within the zebra finch
stages affect both proliferation and serotonergic innervation in the adult rostral
Dietrich, A., & Allen, J. (1999). Functional dissociation of the prefrontal cortex and the
Annual Review of Neuroscience, 22(1), 567-631.
emergence during vocal development. Journal of Neuroscience, 17, 1147–1167.
Cognitive Sciences, 11(12), 504-513.
Dupret, D., Revest, J., Koehl, M., Ichas, F., De Giorgi, F., Costet, P., Abrous, D., Piazza, P.
Neurobiology, 18(2), 131-136.
Enwere, E. (2004). Aging results in reduced epidermal growth factor receptor signaling,
diminished olfactory neurogenesis, and deficits in Fine Olfactory Discrimination.
Journal of Neuroscience, 24(38), 8354-8365.
Ernst, A., Alkass, K., Bernard, S., Salehpour, M., Perl, S., Tisdale, J., Possnert, G.,
attention on late ERP components: II. Error processing in choice reaction
25, 38–46.

Farioli-Veccio, S., Sarauilli, D., Costanzi, M., Pacioni, S., Cinà, I., Aceti, M., Micheli, L.,

Hippocampal Neurons Is Crucial for Spatial Memory. PLoS Biology, 6, 319 - 335.


Fortune, E., Margoliash, D., (1992). Cytoarchitectonic organization and morphology of cells of
the field L complex in male zebra finches (taenopygia guttata). The Journal of
Comparative Neurology, 423(3), 388-404.

Neurosci, 36, 497-503.


Gallistel, C., King, A., McDonald, R. (2004). Sources of variability and systematic error in
mouse timing behavior. Journal of Experimental Psychology: Animal Behavior
Processes, 30(1), 3-16

Garrett, M. C., & Soares-Da-Silva, P. (1992). Increased Cerebrospinal Fluid Dopamine and 3,4-
Dihydroxyphenylacetic Acid Levels in Huntingtons Disease: Evidence for an Overactive


disease: can studying adult neurogenesis lead to the development of new therapeutic

Glass, R., Synowitz, M., Kronenberg, G., Walzlein, J.H., Markovic, D.S., Wang, L.P., Gast, D.,
endogenous neural precursor cells is associated with improved survival. J. Neurosci. 25,
2637–2646.


Gotts, S., Jo, H., Wallace, G., Saad, Z., Cox, R., & Martin, A. (2013). Two distinct forms of
functional lateralization in the human brain. Proceedings of the National Academy of
Sciences, 110(36).


Gribova, A., Donchin, O., Bergman, H., Vaadia, E., Cardoso De Oliveira, S. (2002). Timing of
bimanual movements in human and non-human primates in relation to neuronal activity


Optical controlling reveals time-dependent roles for adult-born dentate granule cells. Nat
Neurosci., 15, 1700–1706.


Imayoshi, I., Sakamoto, M., Ohtsuka, T., Takao, K., Miyakawa, T., Yamaguchi, M., Mori, K.,


Majerová, V., Kalinčík, T., Laczó, J., Vyhnálek, M., Hort, J., Bojar, M., Ruzicka, E., Roth, J. (2012). Disturbance of real space navigation in moderately advanced but not in early


Thompson, J., Wu, W., Bertram, R., Johnson, F. (2007). Auditory-dependent vocal recovery in adult male zebra finches is facilitated by lesion of a forebrain pathway that includes the basal ganglia. *Journal of Neuroscience* 27, 12308-12320.


Wittmann, M., & Wassenhove, V. V. (2009). The experience of time: Neural mechanisms and the interplay of emotion, cognition and embodiment. Philosophical Transactions of the Royal Society B: Biological Sciences, 364(1525), 1809-1813.


