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Adult Neurogenesis in Avian Auditory Cortex, Caudomedial Nidopallium (NCM): Lateralization and Effects of Statins

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ADULT NEUROGENESIS IN AVIAN AUDITORY CORTEX, CAUDOMEDIAL NIDOPALLIUM (NCM): LATERALIZATION AND EFFECTS OF STATINS

by

SHUK C. TSOI

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

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

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THE CITY UNIVERSITY OF NEW YORK
ABSTRACT

Adult Neurogenesis in Avian Auditory Cortex, Caudomedial Nidopallium (NCM): Lateralization and Effects of Statins

by

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Advisor: Dr. Carolyn L. Pytte

In song learning, young zebra finches use the memory of the tutor’s song to produce his own song during the critical learning period. This memory, as well as the memory of conspecific songs, are stored in the secondary auditory cortex called the caudomedial nidopallim (NCM), which is one of a few regions in the avian brain where adult-born neurons are found. Even though the left NCM and the right NCM are functionally different (Phan and Vicario, 2010), the distribution of new neurons between the left and the right NCMs is unknown. In addition, the relationship between the number of new neurons in NCM and learning and memory is unclear. In part 1, we quantified the number of new neurons in both left and right NCMs and correlated this number with song learning and memory. In this work, adult male zebra finches received injections of bromodeoxyuridine (BrdU, ~0.078 mg/g, IM, 3x/day for 3 days) to label mitotically active cells. Thirty days after the final injection, birds’ song were recorded and electrodes were implanted bilaterally into both left and right NCM to measure responses to novel conspecific songs and familiar conspecific songs that the birds were exposed to 20 hours earlier. Brains were then processed with immunohistochemistry (IHC) to label BrdU and the neuron-specific protein NeuN or Hu. Using fluorescence microscopy, we found that most birds had more new neurons in the left NCM than the right NCM (p = 0.005, two-tailed paired t-test). We also found that the relative number of new neurons between the left and the right NCMs, which was...
calculated as a normalized “Neuron Asymmetry Index”, was correlated with song learning ($R^2 = 0.23, p = 0.027$) and auditory memory in the right NCM ($R^2 = 0.31, p = 0.038$). These are the first report of lateralization in neurogenesis, and its functional correlates, in any brain region and in any organism.

Statins, which are approved for children with high cholesterol, can affect brain cholesterol after crossing the blood-brain barrier. Moreover, some adult statin users report cognitive impairment while taking statins, although this claim is controversial. The effects of statins on children’s neural development and cognition are not clear and have not yet been directly studied. In this work, we used juvenile zebra finches to study whether atorvastatin (Lipitor) affects song learning, auditory memory and new neurons. Male juvenile zebra finches were given a daily dosage of either 40mg/kg of Lipitor or water as vehicle for ~50 days throughout the critical song learning period and until a day before sacrifice. These birds were exposed to a standard song playback, via a speaker in their cages during the critical learning period (45 – 80 days post hatched). Before they were sacrificed, the bird’s own song was recorded and the memory of the tutor’s song in NCM was measured electrophysiologically. Brains were processed using IHC and we used Neurolucida to quantify the number of new neurons in NCM and HVC of the motor pathway and to automatically measure the traced contours of the somas of neurons in HVC. Using Sound Analysis Pro 2011, we found that there was a trend that atorvastatin-treated birds had lower quality of song copy than control birds ($p = 0.06$, two-tailed t-tests). We also found that Lipitor-treated birds had weaker memory of the tutor’s song than control birds ($p = 0.048$, two-tailed Mood’s median test). Counts of new neurons in both NCM and HVC were not affected by treatment. However, in HVC, Lipitor affected the morphology of 30-day old neurons, and the size of the older neurons. These changes
suggest that statins affected neurons that are important for and memory storage and song
production during the critical learning period.

Statins can be classified by the degree of lipophilicity. It is thought that lipophilic statins
may affect brain cholesterol more than hydrophilic statins due to the easiness to cross the blood-
brain barrier. In the last part of the dissertation, we asked whether lipophilic simvastatin has a
stronger effect on memory and neurogenesis than hydrophilic pravastatin. Adult zebra finches
were given a daily dosage of either 40 mg/kg of pravastatin, simvastatin or water as vehicle for
60 days. These birds received BrdU injections (~0.078 mg/g, IM, 3x/day for 3 days) to label
new neurons and were sacrificed ~30 days after the last injection. We found that while
simvastatin did not affect song memory, pravastatin-treated birds had a weaker memory than
control birds (Mixed ANOVA, Post-Hoc (LSD), p < 0.05). However, consistent with our
findings with juvenile birds, neither statin affected the number of new neurons in NCM. These
results illustrate that lipophilicity may not be the only determinant of statins’ effects in memory
and neurogenesis.
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CHAPTER 1: BACKGROUND AND LITERATURE REVIEW

General Introduction

The establishment of the field of adult neurogenesis is fairly recent. Before Altman’s discovery in 1962 (Altman, 1962), it was long believed that the brain was different from the other organs in the human body. While cells in other organs constantly undergoing mitosis to replace cells that are damaged and dying, replacement of neurons was not thought to occur. Joseph Altman and colleagues discovered new cells in the brains of adult rats. However, this discovery was not well received since it was unknown whether the new cells were actually neurons (Nottebohm, 2004). It wasn’t until 1983 when Steven Goldman and Fernando Nottebohm discovered new neurons incorporated into functional circuits in the songbird’s brain that adult neurogenesis was recognized. Since then, adult neurogenesis has been studied in nearly all major orders of vertebrates.

In the mammalian brain, new neurons were first discovered in the olfactory bulb and the dentate gyrus of the hippocampus (Kempermann, 2006). More recently, new neurons have been identified in the hypothalamus, amygdala, and piriform cortex (Review: Fowler et al., 2008; Yuan and Arias-Carrion, 2011). Most work has focused on new neurons in the hippocampus, where they are limited to the dentate gyrus. This leads to the question of what function they may serve. New hippocampal neurons have membrane properties that allow them to be easily excited. Moreover, since the hippocampus functions in learning and memory formation, many studies focused on how new neurons may contribute to learning and memory.

Like the mammalian brain, the incorporation of new neurons in the songbird’s brain is not random. In addition to the hippocampus, new neurons are also found in three distinct
interconnected regions in the songbird's brain, which underlie vocal learning and memory. One of these regions is the caudomedial nidopallium (NCM), which is analogous to the mammalian auditory association cortex. In this work, we used the zebra finch as a model organism to study how new neurons in NCM may relate to song learning and memory.

Learning and memory impairments are symptoms of neurodegenerative diseases (such as Alzheimer’s, Parkinson’s and Huntington’s Diseases), which co-occur with altered neurogenesis (Mu and Gage, 2011). Interestingly, statins, which are usually prescribed to lower cholesterol, are known to be neuroprotective, and can alleviate these cognitive symptoms in the diseased brain (Yaffe et al., 2002; Lu et al., 2007; Wu et al., 2008; Yuksel et al., 2013; Robin et al., 2014). Specifically, statins can lower inflammation and increase neurogenesis in diseased and injured brains. However, in addition to these positive properties, statins might be detrimental to learning and memory in healthy brains. Moreover, the effects of statins on the brain may be related to how easily they can cross the blood-brain barrier. To date, findings on the effects of statins in healthy brains are mixed and controversial. Despite these controversies, the Food and Drugs Administration has approved the use of four kinds of statins in children who have genetically-related high cholesterol (Stein, 2007) without an understanding of whether statins affect learning and memory in the developing brain.

The overall goal of this work is to address gaps in our understanding of the potential neural and cognitive effects of statins.

**Adult neurogenesis in mammals**

In the mammalian brain, new neurons arise from the subventricular zones of the lateral and third ventricles, and the subgranular zone of the dentate gyrus. New neurons originating from the lateral ventricles populate the olfactory bulb, amygdala, and piriform cortex whereas new
neurons from the third ventricles migrate to the hypothalamus. Neural precursors formed in the subgranular zone migrate to the granular cell layer of the dentate gyrus (Bernier et al., 2002; Yuan et al., 2015).

The neural precursor cells in all neurogenic regions are in fact radial glia cells and express glial fibrillary acidic protein (GFAP), which is a standard marker for astrocytes (Campbell and Götz, 2002; Whitman and Greer, 2009; c.f. Voigt, 1989; Sancho-Tello et al., 1995). Radial glia in the subventricular zone generate a cell type called “transient amplifying cells” that then generate neuroblasts (Whitman and Greer, 2009), which migrate to the olfactory bulb via a discrete pathway called the rostral migratory stream. The migratory pathways of these neuroblasts corresponds to the flow of cerebrospinal fluid in the lateral ventricles that was generated by ependymal ciliary beatings (Sawamoto et al., 2006). From the center of the olfactory bulb, the neuroblasts use blood vessels as a scaffold to migrate to the periphery of the olfactory bulb (Bovetti et al., 2007) where they differentiate into two types of interneurons: granule cells and periglomerular cells (Lois and Alvarez-Buylla, 1994).

In the hippocampus, the process is slightly different. Instead of producing the intermediary transient amplifying cells, radial glia cells in the subgranular zone of the dentate gyrus generate intermediate progenitor cells that give rise to neuroblasts (Song et al., 2012). Since radial glia cells from different regions have different transcription factors, it is thought that the transient amplifying cells and intermediate progenitor cells have different functional roles (Kempermann, 2006). The hippocampal neuroblasts then migrate a short distance (1-3 mm) to the granular cell layer of the dentate gyrus, where they differentiate into granular cells. Neuronal differentiation in the third ventricle, giving rise to hypothalamic neurons, has not yet been characterized (Maggi et al., 2015).
Young neurons in both the olfactory bulb and dentate gyrus integrate into the existing circuitry (Epp et al., 2007). Initially, young neurons have an excitatory response to tonic GABA activation, which is usually an inhibitory neurotransmitter. At this point, the new neurons are depolarized by an efflux of negatively charged intracellular chloride. Interestingly, this process is essential for dendritic arborization. As the neurons mature, they then become hyperpolarized by phasic GABA innervation (Ge et al., 2006). After this time, depolarization occurs by the activation of glutaminergic inputs.

After migration and differentiation, new neurons then appear to undergo a “critical period” during which they are sensitive to the local microenvironment and circuit activity. During this period, 50% of new neurons in the olfactory bulb generally do not survive (Petreanu and Alvarez-Buylla, 2002). In the hippocampus, 67% of new neurons do not survive. The critical period for neuronal survival is between 14-45 days (Petreanu and Alvarez-Buylla, 2002; Yamaguchi and Mori, 2005; Mouret et al., 2008) in the olfactory bulb, and is approximately 1-2 weeks after birth (Gould et al., 1999) in the hippocampus.

Numerous factors have been shown to influence a new neuron’s survival during this critical period, including associative learning, (Gould et al., 1999; Leuner et al., 2004; Alonso et al., 2006; Mouret et al., 2008), physical activity (van Praag et al., 1999; Brown et al., 2003; Opendak and Gould, 2015), environmental enrichment (Brown et al., 2003; Tashiro et al., 2007), and stress (Schoenfeld and Gould, 2012; Opendak and Gould, 2015). After surviving the critical period, new neurons may live up to various ages. For instance, new neurons in the olfactory bulb can survive for 1 year (Petreanu and Alvarez-Buylla, 2002) and new neurons in the dentate gyrus can survive for several months (Kempermann et al., 2003; Leuner et al., 2004).

**Adult neurogenesis in songbirds**
In birds, neural proliferation occurs in the subventricular zones of the lateral ventricles. However, proliferating cells are not homogeneously distributed, but are found in clusters or “hot spots” (Alvarez-Buylla et al., 1990). The proliferating cells in the hot spots are the neuronal precursors called Type B cells, which are radial glia. These cells then generate Type A cells, which are neuroblasts, that have the characteristics of migratory cells (Alvarez-Buylla et al., 1998).

These Type A cells migrate through regions that are rich in radial glia fibers (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla and Kirn, 1997). Moreover, young neurons also migrate via “tangential migration”, where their paths are perpendicular to the radial glia pathway (Barnea and Pravosudov, 2011), before migrating along the radial cell fibers (Doetsch and Scharff, 2001). However, while bipolar migratory neurons use radial glia for migration, multipolar migratory neurons use neither radial glia nor blood vessels for guidance. They translocate their cell bodies along their own processes to move forward, and change direction by moving the cell body towards one of their own processes that leads to another route. It was also hypothesized from the close proximity of young neurons to mature neurons that the interaction with the mature neurons may be necessary to determine their final destinations (Scott et al., 2012). There may be an increase of reelin signaling from the mature neurons that prevent the young neurons from continuing to migrate. Reelin expression also prevented new hippocampal neurons from migrating into the hilus region (Duan et al., 2008).

Sampling from one telencephalic region, it was determined that only about one third of migratory neuroblasts became neurons, suggesting that most migratory cells die (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla and Kirn, 1997; Barnea and Pravosudov, 2011). New neurons were found throughout the telencephalon but they were only incorporated in three
telencephalic regions that function specifically in song behavior: HVC of the posterior motor pathway of the song system, Area X of the anterior forebrain pathway of the song system, and NCM of the ascending auditory pathway. The song system is the group of interconnected nuclei underlying song learning, perception and production (described fully below in “The song system”). Interestingly, other nuclei in the song system do not receive new neurons.

HVC contains two groups of projection neurons. One group projects to the robust nucleus of the archistriatum (RA), which is the premotor nucleus in the song production pathway. Another group projects to Area X, a striatal nucleus necessary for juvenile song learning and adult song maintenance. Interestingly, only RA-projecting neurons are incorporated during adulthood. In fact, when X-projecting neurons were selectively killed in juvenile birds, the response was a surge in RA-projecting neurons in HVC (Scharff et al., 2000).

The number of new neurons recruited to HVC differs across different species of songbirds (Barnea and Pravosudov, 2011). For birds such as the canary that change their songs seasonally by adding or deleting song elements, the recruitment of new neurons in HVC was shown to correlate with neuronal death, which suggests new neurons are compensating for the death of existing neurons. Moreover, new neurons were primarily added during times of change in song elements, perhaps contributing to new song motor patterns (Kirn et al., 1994). Interestingly, the survival of HVC new neurons for these birds depended on the time of year when the neurons were born. In canaries, neurons that were born in the fall lived longer (~8 months) than those born in the spring (~4 months) (Nottebohm et al., 1994). However, for birds such as the zebra finch that produce only one stereotyped song in their lifetime, the recruitment of new neurons in HVC was not balanced with death of existing neurons (Walton et al., 2012). New HVC neurons in zebra finch HVC survive up to 11 years (Walton et al., 2012).
In Area X, only spiny neurons are replaceable and, interestingly, these neurons express FoxP2, a transcription factor that is involved in human speech development (Rochefort et al., 2007). In NCM, characteristics of new neurons have not been studied.

In addition to these three telencephalic regions, new neurons are also recruited to the avian hippocampus. Like the new neurons in the mammalian hippocampus, avian hippocampal new neurons are associated with spatial memory, for example, used in food-caching and migration (Hoshooley and Sherry, 2007; Barnea and Pravosudov, 2011).

Properties of young neurons

Most of what is known about new neuron electrophysiology has been demonstrated in the mouse hippocampus. These young hippocampal granule cells have properties that contribute to plasticity (Synder et al., 2001; Schmidt-Hieber et al., 2004). Young neurons had fewer primary dendrites, and those they had were thin and varicose, with fewer, shorter, and thinner dendritic branches than dendrites in mature granule cells (Wang, Scott and Wojtowicz, 2000; Spampanato et al., 2012). Young neurons have been shown to lack GABAergic inhibition and have high input resistance (Wang et al., 1999). They demonstrated low threshold spikes, which were generated by T-type Ca^{2+} (low-voltage activated) channels, which make it easier to reach long term potentiation (LTP) (Schmidt-Hieber et al., 2004). Mature granule cells had a higher threshold for action potentials. LTP produced by young neurons (neuron age ≤ 3 weeks old) depended on the activation of NR2B/NMDA receptors and showed easier induction of LTP than did mature neurons (Synder et al., 2001). Moreover, during stimulation, young neurons exhibited paired-pulse facilitation while mature neurons showed paired-pulse depression (Wang et al., 1999). These distinct properties allow young neurons to be activated more easily with
excitatory inputs compared with mature neurons of the same type (granule neurons) and in the same region (dentate gyrus).

**The role of neurogenesis in learning and memory**

There is substantial evidence that adult-born hippocampal granular cells contribute to learning and memory in rodents (Snyder et al., 2005; Tashiro et al., 2007; Deng et al., 2009; Trouche et al., 2009; Aimone et al., 2011; Vukovic et al., 2013; Akers et al., 2014). One of the most convincing reports demonstrated that complete ablation of immature hippocampal neurons prior to training impaired the learning of a spatial task. When the population of immature neurons was replenished, learning recovered (Vukovic et al., 2013). Interestingly, however, when there was only a partial reduction of immature hippocampal neurons, learning was not impaired (Goodman et al., 2010).

A role of new neurons in memory storage can be illustrated by neuronal responses measured by immediate early gene (IEG) expression following exposure to different experiences. Studies showed that new hippocampal neurons had more IEG expression when they were re-exposed to a familiar experience that occurred when the population was young, than when they were exposed to a novel experience (Tashiro et al., 2007; Trouche et al., 2009). The interpretation of this result was that young new neurons encode memories of contexts, and this can be demonstrated when the animal is re-exposed to that same context. Moreover, it was proposed that immature neurons in the hippocampus allow the animal to have higher memory “resolution” so that it can distinguish remembered objects more easily (Aimone et al., 2011). This idea is consistent with the suggestion that partial reduction of immature hippocampal neurons may have resulted in memories becoming less detailed (Goodman et al., 2010).

However, contradictory to these studies, recent work suggests that new neurons may interfere
with storage of old hippocampal dependent memories (Akers et al., 2014). Thus, the role that new neurons play in memory and learning is still unclear.

Other work suggests that there is a critical time window in which new neurons are able to store new information. New neurons appear to encode memory when the neurons are young and not yet fully mature (Trouche et al., 2009). This idea is supported by studies showing that a reduction of young neurons impaired spatial learning and long term memory retention (Snyder, Hong, McDonald, & Wojtowicz, 2005; Deng et al., 2009). However, a reduction of immature neurons that had not yet been incorporated into a circuit did not affect memory retrieval of a familiar spatial task (Vukovic et al., 2013).

**Roles of learning and memory on neurogenesis**

Learning has been shown to increase the survival of new neurons, suggesting that the survival of new neurons can be used to determine the success of learning (Gould et al., 1999; Döbrössy et al., 2003; Trouche et al., 2009). The majority of newborn neurons in the mouse hippocampus die by 2 weeks of cell birth (Cameron et al., 1993; Gould et al., 1999). It was first thought that only hippocampal dependent tasks rescue neurons that would otherwise have died (Gould et al., 1999). However, it was later determined that some hippocampal independent tasks, such as stimulus contiguity conditioning tasks, were able to rescue new neurons while hippocampal dependent tasks such as short trace conditioning tasks did not rescue new neurons (Shors, 2008; Curlik et al., 2013). Moreover, there is a critical period during which neurons can be rescued by learning. Associative learning only affected the survival of 1 week old neurons and had no effect on neurons that were born earlier or later relative to the task (Anderson et al., 2011). It has also been demonstrated that the level of difficulty of the tasks (Curlik and Shors, 2011; Curlik et al.,
2013) and quality of learning (Dalla et al., 2007) are important factors that can rescue neurons from death.

**Song learning**

The process by which songbirds learn their songs is surprisingly similar to human speech acquisition (Doupe and Kuhl, 1999). For instance, both humans and songbirds need to hear others’ vocalizations as well as their own in order to produce normal speech or song (Doupe and Kuhl, 1999). If a child becomes deaf before or during speech development, the speech deteriorates in a similar way as it does in songbirds (Doupe and Kuhl, 1999). Similarly, if a bird is deafened prior to song learning, they will develop distinct abnormalities in song structure, known as “deaf song”. A bird deafened after song learning will show deterioration of the song and produce a non-distinct series of sounds (Doupe and Kuhl, 1999). However, there also appears to be some innate species-specific song structure as deaf songs contain some similarities to normal songs (Price, 1979). Moreover, the later one is deafened (either bird or human) the more song or speech is retained and the slower the degradation (Lombardino and Nottebohm, 2000).

In adult songbirds, auditory feedback still plays a role in maintaining song stability after song learning if complete. This is seen when adult zebra finches were deafened and song structure gradually changed over the course of about 6-8 weeks after deafening (Nordeen and Nordeen, 1992; Wolley and Rubel, 1997; Leonardo and Konishi, 1999; Pytte et al., 2012). However, the speed of deterioration varies among individual birds (Lombardino and Nottebohm, 2000; Pytte et al., 2012) and across species (Wolley and Rubel, 1997). Auditory feedback is also used to maintain the stability of speech in adult humans (Waldstein, 1990) and perhaps similar mechanisms of sensory-dependent motor maintenance are used in birds and humans.
Social experience is also important for both speech and song learning. In addition, for both types of vocal learning, there is a critical (or sensitive) period during which speech and song must be learned to ensure normal vocalizations. These remarkable similarities allow us to use songbirds as a model system to study neural aspects of learning and memory underlying vocal learning. Moreover, variables that contribute to song learning are easy to manipulate and success in learning is quantifiable, thus lending the songbird model to experimentation (Tchernichovski et al., 2000).

Song learning occurs in two phases: the sensory and the sensorimotor periods, which, in zebra finches, occur during overlapping critical periods (Pytte and Suthers, 2000; Brainard and Doupe, 2002). During the sensory period, the young bird listens to songs produced by adult males, and memorizes elements of these song models. During the sensorimotor period the young bird practices singing the adult song and increasingly imitates song elements.

There are numerous species differences in song learning, including whether these phases overlap or are separated in time, the time between these phases, the number of sensory and sensorimotor phases, the song properties that are susceptible to change during song learning and the amount of adult model song exposure necessary for successful learning (Konishi, 1985; Tchernichovski et al., 1999).

In this work, we use the zebra finch, which is a “closed-period” learner, capable of song learning only once, during a juvenile developmental period. Zebra finches learn a single highly stereotyped song, which they repeat unchanged throughout their life. In zebra finches, the sensory phase occurs from about 20 to 60 days of age and the song model that is copied is usually the song of the juvenile’s father, referred to as the “tutor”. If exposure to adult song does
not occur within this time frame, successful learning will not occur (Bohner, 1983). If the bird is raised in isolation, it will develop an “isolate song,” which contains aberrant song elements.

In zebra finches, the sensorimotor phase overlaps that of the sensory period, and occurs between days 25 to 90 days of age. In the beginning of the sensorimotor phase, the juvenile produces highly variable sounds called “subsong”, which are low amplitude, and have a wide and variable frequency range (Böhner, 1983). Subsong gradually becomes more stable and the general song organization becomes more distinct (Konishi, 1985). It is then considered to be in the “plastic” song stage. It is thought that the bird continuously compares his own song to the memory of the tutor’s song, and in this way his song becomes increasing similar to that of the tutor (Solis and Doupe, 2000; Mooney, 2009). However, the juvenile rarely produces an identical copy of the tutor’s song. Instead, individual modifications include removing, changing, or adding song elements resulting in a unique song. At approximately 90 days of age, the song is said to become “crystallized” and is highly stereotyped (Morrison and Nottebohm, 1993; Solis and Doupe, 2000). After crystallization, the bird can no longer modify his song.

For the highly social zebra finch, song learning is more accurate when interacting with a live tutor (Deregnaucourt et al., 2013). However, direct interaction with an adult bird is not essential for song learning. Young birds can also learn to sing a recorded song played back from a speaker, but only when the playback is interactive and the juvenile “controls” song exposure (Adret, 1993; Tchernichovski et al., 2000).

**The song system**

Song learning and production require a set of interconnected nuclei called the “song system”. The song system is divided into the anterior forebrain pathway (AFP) and the posterior motor pathway. The ascending auditory pathway feeds into the song system at the intersection of the
AFP and the motor pathway at the nucleus HVC (used as a proper name), which projects to both pathways. In zebra finches, the two hemispheres are only connected through the inter-hemispheric commissure (Ashmore et al., 2008). The song system regions in both hemispheres are anatomically the same, as far as has been determined.

**Anterior Forebrain Pathway**

The AFP functions in the acquisition of song and modulation of song plasticity. In this pathway, neurons in HVC project to nucleus Area X, which in turn project to the dorsomedial nucleus of the thalamus (DLM). These thalamic neurons project to the lateral magnocellular nucleus of the anterior nidopallium (LMAN). Neurons in LMAN project back to Area X, and also to the poster motor pathway robust nucleus of the arcopallidum (RA, Figure 1A). Area X is homologous to the mammalian basal ganglia, based on neuron types and their projections to the thalamus (Gale and Perkel, 2010). During juvenile singing, neurons in the AFP are activated and are thought to monitor singing motor feedback, which is one function of the mammalian basal ganglia. One model of song learning proposes that Area X and LMAN function in determining the degree of similarity between the bird’s own song and that of the memorized tutor’s song. This comparison results in generating “error signals” that drive subsequent song development (Brainard and Doupe, 2000). During song learning, neural tuning in the AFP changes in a way that supports this model. During the sensory phase, neurons in the AFP are not selective, and show equal responsivity to playback of the tutor’s song, songs of other conspecifics, and the tutor's song played in reverse as a control for song-like auditory features (Doupe and Solis, 1997). During the sensorimotor phase, neurons in the AFP become tuned first to the tutor’s song, and then as the song becomes more developed, they became more selective for the bird’s own song (Doupe and Konishi, 1991; Solis and Doupe 2000; Roy and Mooney, 2007). In the adult brain, LMAN
and Area X neurons are still tuned to the bird’s own song, and perhaps this plays a role in monitoring and maintaining song stability based on auditory feedback during singing (Doupe and Solis, 1997).

Dorsal RA, which is involved in respiratory control, projects to the posterior portion of the dorsomedial thalamic nucleus (DMP), which innervates the medial magnocellular nucleus of anterior neostriatum (mMAN). mMAN then projects back to HVC (Vates et al., 1997). It was suggested that this thalamo-telencephalic loop is important for the temporal organization of the song (Vates et al., 1997).

Posterior Motor Pathway
The posterior motor pathway is necessary for the production of song. In this pathway, neurons from HVC project to RA. Death of HVC-to-RA projection neurons in adult birds caused crystallized songs to deteriorate (Scharff et al., 2000). RA also innervates the tracheosyringeal portion of the nucleus hypoglossus (nXIIts) that directly innervates the muscles of the syrinx, which is the avian vocal organ (Figure 1A).

Ascending Auditory Pathway
Sound first activates the cochlear ganglion, which sends the signal to the midbrain nucleus mesencephalicus lateralis dorsalis (MLd). Nucleus ovoidalis of the thalamus receives innervation from MLd and sends projections to the primary auditory cortex, Field L. Field L consists of three parts: Field L 1, Field L 2 (which is divided into Field L 2a and 2b), and Field L3. These components also communicate with each other: Field L 2a has reciprocal innervations with Field L 2b, Field L 1, and Field L 3. The signals from each component of Field L are then sent to the secondary auditory cortices, the caudomedial nidopallium (NCM) and the
caudomedial mesopallium (CMM), which also receives reciprocal signals from Field L1, Field L2b and Field 3. Field L2a and Field 3 directly project to NCM. NCM and CMM also interact with each other. In addition, CMM sends inputs to HVC and song nucleus interface (NIf) (Bauer et al., 2008, Figure 1B). Neurons in the entire ascending auditory pathway are predominately GABAergic, suggesting that inhibitory mechanisms are important for auditory processing (Fujita and Konishi, 1991; Yang et al., 1992; Pinaud and Mello, 2007).

**Measurements of memory in NCM**

In order to learn to sing a normal song, the juvenile must form an accurate memory of the tutor song. Electrophysiological recordings and IEG expression studies have shown that NCM is a site for long-term storage of the tutor’s song, historically referred to as the tutor song “template” (Phan et al., 2006; Bolhuis et al., 2000).

ZENK, which is the homolog of mammalian egr-1, is an IEG that is highly conserved across different species (Long and Salbaum, 1998), and can be used as an indicator of neuronal activity, and may play a role in the consolidation of memory (Jarvis, 2004). When adult male birds were re-exposed to their tutor song, ZENK expression in NCM increased. Moreover, birds that had songs that were similar to the tutor’s songs had greater ZENK expression in response to the playback of the tutor’s songs (Bolhuis et al., 2000). Interestingly, this suggests that the strength of the memory for the tutor’s song corresponds to the quality of song learning.

In addition to memory storage of the tutor’s song, NCM also stores memories of other conspecific songs. Playback of conspecific songs induced significantly higher levels of ZENK in NCM than heterospecific songs or tones (Mello et al., 1992). ZENK expression patterns result in an intriguing phenomenon: ZENK expression in NCM is very high when a bird hears a new conspecific song and expression decreases with subsequent exposure to the same song (Mello et
This decrease suggests that the change in gene expression may be related behaviorally to recognition memory for the song, and thus has been used as a tool to assess memory.

Similar to ZENK studies, electrophysiological studies can be used to study learning and memory at a neuronal level. In this context, learning and memory are indicated by the neuronal firing rate and the adaptation rate of the population of NCM neurons in response to song playbacks. When a bird initially hears a novel song, the population activity level of neurons is high. As the bird continues to listen to the same song, neural activity gradually decreases (Chew et al., 1995, 1996; Phan et al., 2006). The adaptation rate is the slope of decrease in neuronal response to the repeated playback of a single song (Phan & Vicario, 2010). Thus the bird’s “familiarity” with the song can be illustrated by the neuronal adaptation rate (Chew et al., 1996).

Using this technique, a weak neuronal response and low adaptation rate is interpreted to indicate a strong memory (Phan and Vicario, 2006). It has been shown that NCM neurons remain adapted to a given conspecific song up to 48 hours (Chew et al., 1995). Zebra finches remain familiar with the tutor’s song after 30 days without song exposure, suggesting a long term memory for the song that was encoded during an early critical period (Phan et al., 2006). Longer time periods have not been tested.

It is interesting to note that although adapted, NCM neurons will again fire strongly to new song stimuli. Moreover, NCM neurons differentially store memories of multiple songs. The adaptation is specific to familiar stimuli regardless of whether there are different song stimuli or periods of silence in between presentations of the familiar song (Chew et al., 1996). It was suggested that NCM neurons interact with each other in multiple “ensembles” and this interaction results in song-specific adaptation to familiar stimuli (Chew et al., 1995). It is
possible each neuron can modulate its genetic response to a specific stimulus within each
ensemble to elicit adaptation (Mello et al., 1995). This ability to produce different responses
may indicate that each ensemble has a different degree of involvement in learning various songs
(Mello et al., 1995).

**Neurogenesis in NCM and HVC**

In HVC, adult-born neurons seem to be important for song learning (Krn et al., 1991) as well as
song stability and the maintenance of song structure (Scharff et al., 2000; Pytte et al., 2012).
New neurons that are incorporated into NCM throughout adulthood can be influenced by
functional activity in NCM. For instance, adult neurogenesis in NCM was decreased in birds
that were deafened (Pytte et al., 2010). In addition, adult neurogenesis was increased in birds
living in large groups, consisting of a complex social environment (Lipkind et al., 2002; Barnea
et al., 2006; Adar et al., 2008). Presumably this increase was due to increased song processing.
Interestingly, the survival of the older neurons that were already integrated into an existing
circuit was decreased in the complex environment (Adar et al., 2008). Moreover, the number of
other zebra finches in the living environment affected the distribution of new neurons in NCM.
Birds living in large groups had more new neurons in the caudal end of NCM than in the rostral
end (Adar et al., 2008), suggesting potential regional differences in processing task and/or effects
on new neuron survival. These findings suggest that the new neurons in NCM are needed to
process multiple songs and to store those memories. However, since many studies on the
functions of new neurons are correlational studies, it is not clear what role that new neurons have
in learning and memory.

**Lateralization**
Lateralization describes brain functions that are localized in particular regions in one hemisphere. For instance, in humans, Broca’s area is involved in speech production while Wernicke’s area is involved in speech perception, and both of these regions are usually lateralized in the left hemisphere in right-handed people (Shtyrov et al., 1998). Also, the degree of left handiness is related to the incidence of right hemispheric dominance in language processing (Knecht et al., 2000).

In songbirds, song perception is lateralized as well. The right and left hemispheres respond differently to the bird’s own song, the tutor’s song and conspecific songs (Poirier et al., 2009; Voss et al., 2007; Phan and Vicario, 2010; Moorman et al., 2012; Moorman et al., 2015). Electrophysiological recordings demonstrated that in adult zebra finches, the right hemispheric NCM did not have as strong memory of conspecific songs (including the bird’s own song and tutor’s song) as the left hemisphere (Phan and Vicario, 2010).

In awake normal juvenile zebra finches, there were more ZENK-expressing neurons in left hemispheric HVC to playbacks of tutor’s song and other conspecific songs but this lateralization is shifted to the right hemisphere when the birds were asleep (Moorman et al., 2015). Moreover, for juvenile birds that were asleep, the lateralization of neuronal activation was related to the quality of song learning such that “good” learners had more ZENK-expressing neurons in the left hemisphere while “poor” learners had more ZENK-expressing neurons in the right hemisphere (Moorman et al., 2015). These results demonstrate that the left and right hemispheric NCM are involved in separate functions. In zebra finches, the control of song production is somewhat lateralized and dominant in the right hemisphere (Williams et.al., 1992).

**Use of the songbird model system to test the effects of statins on learning and memory**
Songbirds are one of a small group of terrestrial animals to learn their vocalizations, and as such are used as a model system to investigate mechanisms of vocal sensorimotor learning and memory. They are also important animal models for studying critical period vocal learning. In the following work, we are using the songbird model system to investigate the potential effects of statins, which are commonly prescribed cholesterol-lowering drugs, on learning and memory. It was reported that statins treatments decreased serum cholesterol level in zebra finches (McGraw and Parker, 2006) and in hens (Elkin et al., 1999). The reduction was reversed when the birds were given dietary cholesterol supplement along with statins (McGraw and Parker, 2006). Statins can lower not only serum cholesterol levels (manufactured in the liver) but also affect brain cholesterol, which is produced independently in the brain, after crossing the blood-brain barrier (Locatelli et al., 2002; Kirsch et al., 2003; Burns et al., 2006). Adult users have reported memory loss related to their statin treatments, and symptoms of cognitive impairments were alleviated upon the termination of the treatment (Wagstaff et al., 2003). However, findings from research on the cognitive effects of statins are mixed. Furthermore, neurons and glia treated with statins in vitro had damaged structures and altered cellular morphology. However, it remains to be determined whether statins affect neurons and glia in vivo the same way as in cultured cells.

Statins and children

Despite the potential effects of statins on learning and memory, in 2002, the Food and Drug Administration approved giving statin drugs (lovastatin, simvastatin, pravastatin, atorvastatin) to children as young as 8 years old who have familial hypercholesterolemia, a genetic disorder characterized by high cholesterol (Stein, 2007). However, the effectiveness of statins to treat familial hypercholesterolemia in children was mostly extrapolated from the results of studies of
adults (O’Gorman et al., 2011). While many studies with children focused on short-term effects of statin treatment on physical growth and sexual development (de Jongh et al., 2002; Lamaida et al., 2013), research on how statins affect children’s cognition and neural development was limited to their school performance, which is not a very detailed assessment (Herman, 1992).

**Brain cholesterol**

Statins decrease the production of cholesterol in the liver by inhibiting HMG-CoA reductase, a rate-limiting enzyme for cholesterol synthesis in the cell (Figure 2). Brain cholesterol is created *de novo* by astrocytes and oligodendrocytes and is produced independently from blood cholesterol (Saheb et al., 2005; Funfschilling et al., 2007). Neurons can also produce cholesterol but synthesis is much slower and less efficient than in glia cells as shown by *in vitro* studies (Nieweg et al., 2009). Mature cortical neurons and embryonic neurons primarily rely on astrocytes and microglia to supply cholesterol respectively (Funfschilling et al., 2007).

**Effects of statins in learning and memory**

The effects of statins on learning and memory in adults are unclear, and findings are contradictory (Wagstaff et al., 2003; Baytan, et al., 2008; Bettermann, et al., 2012; Maggo and Ashton, 2014; Ott, et al., 2015). While some statin users have reported problems with memory (Wagstaff et al., 2003), others have found no relationship between statins and cognitive impairments in healthy individuals (Ott et al., 2015), in people with mild cognitive impairments (Bettermann et al., 2012), and in people with Alzheimer’s disease (Ott et al., 2015). Due to the potential effects of statins on cognition, in 2012, the FDA required all statins to carry a warning label advising users about the possible risks (Sahebzamani et al., 2014).
According to one animal model study, whether statins affect memory depends on the dosages. Administration of 10 mg/kg of simvastatin to young rats resulted in more errors and a longer time to navigate through a maze, showing impairment of the rats' spatial memory. A low dosage of simvastatin (2 mg/kg) and atorvastatin (1 mg/kg) also impaired working memory and diminished LTP in guinea pigs (Maggo and Ashton, 2014). However, higher dosage (30 mg/kg) of simvastatin had no effect on memory (Baytan et al., 2008). It is possible that the brain utilizes other mechanisms to counter the effects of higher dosage of simvastatin.

Statins can cross the blood-brain barrier, which results in a reduction in brain cholesterol (Locatelli et al., 2002; Kirsch et al., 2003; Burns et al., 2006). Different statins differ in the level of lipophilicity, which is related to the ability to cross the blood-brain barrier (Table 1). Lipophilic statins such as simvastatin (Zocor®) can cross the blood-brain barrier easily (Sierra et al., 2011) and have an effect on cholesterol synthesis (Thelen et al., 2006). However, interestingly, according to one study, simvastatin had no effect on the total amount of brain cholesterol (Thelen et al., 2006), but reduced brain cholesterol concentration in another study (Vecka et al., 2004). On the other hand, hydrophilic pravastatin did not affect cholesterol synthesis in the brain (Thelen et al., 2006), but did lower brain cholesterol (Vecka et al., 2004), and possibly entered the brain via organic anion transporters (Fujii et al., 2015) and a monocarboxylic acid transporter (Tsuji et al., 1993). Due to its hydrophilic structure, pravastatin reduced cholesterol in the outer layer instead of the inner layer of the phospholipid bilayer (Kirsch et al., 2003). For atorvastatin, which is amphiphilic (intermediate lipophilicity), it is uncertain whether it can pass through the blood-brain barrier by simple diffusion and reports are contradictory (Shobab et al., 2005; Sierra et al., 2011).
Studies have suggested that lipophilic statins may affect cognition more than hydrophilic ones perhaps due to their ability to cross the blood-brain barrier easily (Bettermann et al., 2012; Sahebzamani et al., 2014). One longitudinal study reported that only lipophilic statins were associated with the reduced risk of cognitive decline in cognitively healthy people (Bettermann et al., 2012). Contradictory to this report, it was shown that amphiphilic atorvastatin and lipophilic simvastatin were associated with greater risks of cognitive impairments than hydrophilic rosuvastatin and pravastatin (Sahebzamani et al., 2014). Interestingly, however, lipophilicity may not be a major factor determining the drug’s effects on learning and memory. Equivalent doses of pravastatin (low lipophilicity) and not atorvastatin (moderate lipophilicity) impaired learning and memory in adult rats (Stuart et al., 2013).

The cognitive effects of statins might be related to effects on cholesterol at a cellular level. In neurons, cholesterol can be found in the myelin sheath and cell membranes (Bjorkhem and Meaney, 2004). There is uneven distribution of cholesterol in the plasma membrane. It is mostly distributed in the inner layer of the phospholipid bilayer of the plasma membrane, making it less fluid than the outer layer of the membrane (Wood et al., 2011). The reduction of cholesterol by statins in cultured neurons impaired exocytosis of synaptic vesicles (Linetti et al., 2010), which in vivo could would decrease the release of neurotransmitters that are important to learning and memory. Atorvastatin-associated impairments of spatial learning and memory were correlated with the decrease of presynaptic proteins that are involved in the docking of vesicles and necessary for release of neurotransmitter (Schilling et al., 2014; c.f. Parent et al., 2014). This suggests a possible mechanism of how statins might affect cognition at the cellular level.

**Neuroprotective properties of statins in damaged tissue**
Statins have neuroprotective properties for diseased and injured brains. They have been found to alleviate symptoms of traumatic brain injury (TBI) by reducing the level of nitric oxide and vascular endothelial growth factors (VEGFs), which reduced the blood-brain barrier permeability and ischemic damage (Yuksel et al., 2013). They also reduced the levels of inflammatory responses from astrocytes and microglia in animal models of multiple sclerosis, traumatic brain injuries, stroke, and Alzheimer’s disease (Paintlia et al., 2005; Li et al., 2009; Saito et al., 2014; Kurata et al., 2012). Interestingly, the reduction of inflammatory responses increased growth and survival of oligodendrocytes that are involved in the restoration of myelination (Paintlia et al., 2005). The reduction of cholesterol by statins also lowered glutamate-related neurotoxicity seen in TBI and stroke models (Krisanova et al., 2012). Statins can counteract the cognitive impairments associated with these injuries, diseases, as well as old age (Yaffe et al., 2002) by decreasing cell death in the CA3 region of the hippocampus and increasing neurogenesis in the dentate gyrus of the hippocampus (Lu et al., 2007; Wu et al., 2008). The above-mentioned neuroprotective mechanisms could also explain the lower prevalence of Alzheimer disease seen in statin users (Wolozin et al., 2000).

**Neurotoxicity of statins in healthy tissue**

Despite their positive effects in diseased or injured brains, statins may have detrimental effects on healthy brains. *In vitro* studies demonstrated that statin-treated neurons had damaged organelles, fragmented neurites (Pavlov et al., 1995; Michikawa and Yanagisawa, 1999) and compacted nuclei with condensed chromatin (Pavlov el al.; 1995; Tanaka et al., 2000). The toxicity of statin treatments led to an increase in cell death of cortical neurons (Michikawa and Yanagisawa, 1999; Tanaka et al., 2000) and cerebellar neurons (Marz et al., 2007; Xiang and Reeves, 2009).
In addition to their effects on neurons, statin treatments also damaged cell membranes, increased the number of lysosomes (Pavlov et al., 1995), altered cellular morphology and decreased survival of astrocytes, microglia (Marz et al., 2007) and oligodendrocytes (Xiang and Reeves, 2009). Moreover, lipophilic simvastatin and lovastatin were shown to be 10 times more neurotoxic than hydrophilic pravastatin, presumably (but not definitively) due to differences in permeating the blood-brain barrier (Tanaka et al., 2000).

**Statins and neurogenesis**

Neurogenesis is important to learning and memory; therefore, statins’ effects on neurogenesis may be one of the reasons underlying the memory loss suffered by some statins users. Theoretically, statins can directly affect the two neuronal structures that are largely composed of cholesterol: the neuronal membrane and myelin that surrounds the axon. Reducing brain cholesterol can also potentially affect neurogenesis because the new neurons are still developing therefore sequestering cholesterol. However, contrary to these ideas, it was shown that treating healthy mice orally with 10 mg/kg of simvastatin for a week *promoted* neurogenesis in the dentate gyrus via the Wnt signaling pathway (Robin et al., 2014). However, since brain cholesterol has a half-life from 6 months to 5 years, which is much longer than the half-life of blood cholesterol (McFarland et al., 2014), it is possible that the long term effects of statins on neurogenesis would differ from that reported by Robin et al. (2014).
CHAPTER 2: GENERAL METHODS

Animals

All procedures were approved by Institutional Animal Care and Use Committees at Queens College, CUNY, and Rutgers University. Male zebra finches (*Taeniopygia guttata*) of known ages were used for all of the experiments (total n = 72). They were bred and raised in family cages with parents and siblings, and housed in a general aviary room at Rutgers University. For aims 1, 2 and 4, we used adult birds (aims 1 and 2, n = 21 total; aim 4, n = 34 total) that were 82 - 480 days old (mean = 213 days, SEM = 20.6). Zebra finches are considered adult at 80-90 days of age. For aim 3, we used juvenile birds (n = 17 total) that were 18 – 49 days old (mean = 37.4 days, SEM = 2.26) at the start of the experiment. Birds were either tutored by their fathers (aims 1 and 2, n = 14) or by a tape recorded unrelated adult song (aims 1 and 2, n = 4; aim 3, n = 17; aim 4, n = 34). All of the birds in aims 3 and 4 were tape-tutored.

Tutoring

*Father-tutored birds*

Father-tutored birds remained in family cages with both parents and siblings until after the critical period for song learning, > 90 post-hatch day (phd), at which time they were housed in all male groups of 8-10 until the time of the experiment and were then housed single.

*Tape-tutored birds*

When the tape tutored birds were 10 days old, the fathers were removed from the cages. The subject birds were moved from their family cages to individual housing in sound attenuated chambers when they could feed on their own, at 35-40 phd. Sound attenuated chambers were
outfitted for interactive song learning as in Tchernichovski et al., (2000). They contained a microphone to record all vocalizations, a speaker for song playback, a plastic model adult male zebra finch (which facilitates song learning) and two behavioral keys. The juveniles were exposed to the playback of a tutor song, which was a digitized song of an unrelated adult male zebra finch, i.e., “Samba,” from the Sound Analysis Pro (SAP) song file. The same song was used for all tape-tutored birds for standardized comparisons of learning. The song was automatically played when the bird pecked either one of the two keys, and was only played for a maximum of 20 playbacks per day (as in Phan et al., 2006). Subsequent key pecks were allowed but did not result in song playback. When the tutoring period ended at approximately 85 phd, the birds were kept in the isolation chamber for about 30 days so that tests of responses to playback of the tutor song reflected at least a 30-day old memory.

**Song Analysis**

Song learning (aims 1 and 3) was calculated using software designed specifically for this assessment in zebra finches: Sound Analysis Pro 2011(SAP, Tchernichovski, 2000). The adult, crystallized song of the birds (“learned song”) was compared with their tutor’s song (Samba or their father’s song) to assess accuracy of copying. A sample of 10 learned songs was compared with a sample of samba (all songs are identical) or a sample of 100 of the father’s song. Sound Analysis Pro (Tchernichovski, 2000) quantifies the spectral similarity of pairs of songs (in this case, learned song and the tutor’s song) using a sliding 10 ms frame across sound pairs based on the acoustic features: pitch, FM, AM, Wiener entropy, and goodness of pitch (SAP 2011 Online Manual). To produce an overall score of similarity, units for each feature are transformed to units of median absolute deviation from the mean (MAD). Three scores of song imitation were produced. The “Percentage Similarity” is the percentage of the compared song files that pass a
given threshold of similarity. “Accuracy” is a measure of the degree to which similar song segments are alike. “Sequence Match” is a measure of the degree to which notes or song elements appear in the same order across song pairs. The overall Similarity Index is the combination of 2 components: Percentage of Similarity and Accuracy (SAP 2011 Online Manual). The Similarity Index was compared with new neurons in NCM.

**Cell labeling**

All birds received intramuscular injections of bromodeoxyuridine (BrdU; 78 μl of a 10 mg/ml solution in 0.1 M tris buffered saline, pH = 7.4; Sigma) 3 times/day for 3 consecutive days to label mitotically active cells. BrdU is available to dividing cells for approximately 2 hours after systemic injection; therefore, with this protocol, we produced approximately 18 hours of cell labeling. 23 -27 days after the last BrdU injection, electrophysiological recordings were made in NCM to assess memory for previously heard songs (described below). The birds were sacrificed 1 to 3 days after recording. All perfusions occurred 28 +/- 2 days after the last BrdU injection to label 29 – 33 day old neurons.

**Electrophysiological Recording**

Recordings were done at Rutgers University. All recordings were made when the zebra finches reached adulthood, which is about 100 -110 phd. At least 24 hours before recording, birds were anesthetized with nembutal (50 - 55 mg/kg, Abbot Laboratories, North Chicago, IL) or isoflurane in oxygen (2%, Aerrane®, Baxter, Deerfield, IL, USA). A head fixation pin was surgically implanted over NCM and adhered to the skull using dental cement (Dentsply Caulk, Milford, DE). Birds were then placed in their home cage for at least 24 hours to recover before testing.
Testing occurred inside a large acoustically isolated sound booth (IAC, Bronx, NY). For all recordings, the birds were awake and constrained in a custom made holding tube, and the head fixation pin was clamped to a stereotaxic frame. In exploratory penetrations, white noise stimuli with the amplitude envelope of zebra song were used to search for responsive sites from each of the electrodes (Chew et al, 1995,1996; Phan et al, 2006). A motorized micro-drive (Eckhorn, Thomas Recording, Giessen, Germany) was used to independently advance 16 micro-electrodes (quartz platinum/tungsten; impedance 1-3 megohms) into NCM. 8 electrodes were placed bilaterally in the left and right hemispheres to simultaneously record the extracellular multi-unit activity. Signals were amplified (total gain: 19,000), bandpass filtered (0.5 - 5 kHz) and digitized (25 kHz per channel) (as in Phan and Vicario, 2010). Specialized software (Spike 2, Version 7, CED, Cambridge, UK) was used to deliver sound stimuli and record neural activity. All stimuli were the same loudness (played back at 65 dB). The stimuli were played via a speaker located 0.5 meter in front of the bird. Details of playback procedures and analyses of neural responses are described in Specific Methods sections of relevant chapters.

**Histology**

One day after electrophysiological recording, birds were deeply anesthetized with nembutal and perfused transcardially with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The brains were post fixed for 1 h in 4% paraformaldehyde, rinsed in PBS overnight and stored at 4 ºC. Brains were brought to Queens College that night or the following morning and kept at 4 ºC. After rinsing overnight in PBS, the brains were cut along the midline into two hemispheres and dehydrated in ethanol 50% (1 hour), 70% (1 hour), 95% (1 hour) and 100% (40 minutes), and embedded in polyethylene glycol (molecular weight (MW): 1500) (PEG; Polysciences, Warrington, PA). The embedding procedure is a three day incubation
protocol at 47-52 °C as follows: day 1: 1 hour in MW 1000 PEG, 2 hours in fresh MW 1000 PEG, overnight in MW 1000 PEG; day 2: 3:1 (MW 1000:1500) in the morning and 3:1 (MW 1500:1000) in the evening through overnight; day 3: MW 1500 for 30 minutes then embed with MW 1500 PEG in an embedding mold. All brains were stored in a container with desiccants (Drierite™) at 4 ºC. Six-µm sagittal sections were then cut using a rotary microtome. Each hemisphere was sectioned from the medial region to the lateral region. The first complete section through the telencephalon and subsequently every eighth section was mounted onto Superfrost + slides, air dried overnight, and stored at -20 ºC before processing for immunocytochemistry (as in Pytte et al, 2010).

**Immunocytochemistry**

**BrdU**

BrdU plus Hu or Neu-N labeling is a 3-day protocol. During the first day, the slides were brought to room temperature and submerged in citrate buffer at 90-95 ºC for 10 minutes. Then the slides were put in 37 ºC phosphate buffer (PB) for 5 minutes. They were then incubated in 0.25% pepsin in 0.1N HCl at 37 ºC. This enzymatic reaction was stopped when the slides were put in cold PB for 5 minutes followed by two 5 minute PB rinses. After the PB rinses, all the sections were blocked with 10% donkey serum (Jackson ImmunoResearch, West Grove, PA) mixed with 0.3% Triton X and PB for one hour. Then the sections were incubated overnight with primary sheep anti-BrdU in block at 4 ºC (Capralogics, Hardwick, MA). On the second day, the sections were rinsed three times with PB for 10 minutes each. The sections were then blocked with avidin and biotin block (Vector Laboratories, Burlingame, CA) for 30 minutes each. For the next two hours, the sections were incubated in biotinylated donkey anti sheep IgG
(1:200 in 10% donkey serum mixed with 0.3% Triton X and PB, Chemicon International, Temecula, CA). Alexa 488 conjugated to strepavidin (1.25 µg/ml in PB, Molecular Probes, Eugene, OR) was applied to each section for one hour in the dark. After three 10 minute PB washes, the sections were blocked with 10% donkey serum mixed with 0.3% Triton X and PB for one hour. Then the sections were incubated overnight with primary antibody to the neuronal protein Hu (1:10, Invitrogen, Carlsbad, CA) or Neu-N (1:1000, EMD Millipore, Billerica, MA) at 4 ºC. Day 3 started with three 10 minute PB rinses. The sections were blocked with 10% donkey serum in 0.3% Triton X and PB for one hour, then incubated in secondary anti-mouse IgG conjugated to Cy-3 (6.25 µg/ml in PB, Jackson ImmunoResearch, West Grove, PA) for one hour. After three 10 minute PB rinses, all sections were dehydrated as follows: 10 seconds in water, 30 seconds in 50% ethanol, 30 seconds in 70% ethanol, 1 minute in 95% ethanol, 1 minute in 100% ethanol, 2 minutes in xylene. The sections were then cover slipped with Krystalon (as in Pytte et al, 2010).

**Doublecortin (DCX)**

During the first day of immunohistochemistry to label DCX, the slides were brought to room temperature and washed in tris buffered saline (TBS) for 10 minutes. Then they were incubated in a hydrogen peroxide solution (2% hydrogen peroxide, 97% TBS, 1% of methanol) for 30 minutes to quench endogenous peroxidases. After three 5 minutes TBS rinses, slides were blocked with 3% normal horse serum and 2.5% Triton X-100 in TBS for 30 minutes at room temperature. Then the slides were incubated in primary anti-doublecortin made in goat (1:150, Santa Cruz Biotechnology, Dallas, TX) in 3% block at 4 ºC for about 36 hours. On the third day, slides were first rinsed with TBS for three times (5 min each) then incubated with biotinylated horse anti-goat antibody in TBS (1:200, Vector Laboratory, Burlingame, CA) for 3 hours. After
the slides were rinsed in TBS for three times for 5 minutes each, they were incubated in ABC solution prepared from the ABC Elite Kit (1 drop of Reagent A and B for every 2.5 mL of TBS, Vector Laboratory, Burlingame, CA) for an hour. The slides were rinsed again with TBS 3 times for 5 minutes each. Then the slides were incubated 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, all 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 minutes in 100% ethanol, two 15 minutes in xylene. The sections were then cover slipped with Krystalon (Harleco, EM Science, Gibbstown, NJ).

**Mapping NCM**

Data were collected without knowledge of bird’s identity, hemisphere, or treatment. A computer-controlled fluorescence microscope (Olympus BX51) and mapping software (Lucivid microprojection and Neurolucida, Microbrightfield Bioscience Inc.) were used to collect data. The caudal and ventral edges of the brain determine the caudoventral boundary of NCM (Mello et al, 1994). The caudal medial mesopallium (CMM, also known as caudal medial hyperstriatum ventral, CMHV) is adjacent to the rostral edge of NCM in medial sections and demarcated by a visible lamina. In more lateral sections, the rostral border of NCM was defined as 300 µm caudal to the Field L Complex subdivision L2, which is identified using dark field optics and appears as a diffuse bright band of densely packed cells rich in neuropil (Fortune and Margoliash, 1992; as in Pytte et al., 2010).

**Mapping HVC**
Data were collected without knowledge of birds’ identity and treatment, and was only collected from the left hemisphere (see Chapter 6). A computer-controlled fluorescence microscope (Olympus BX51) and mapping software (Lucivid microprojection, Neurolucida) were used to calculate the size of HVC and collect data. HVC was identified by the location of the hippocampus, cerebellum and RA under dark field. Cells in HVC were counted in 10-12 sections from ~1600 to 2200 µm lateral to the midline of each hemisphere.

**Cell Quantification**

BrdU+ cells were identified under a fluorescein isothiocyanate (FITC) filter. Hu or Neu-N labeled cells were viewed with a rhodamine filter. Double-labeled cells were identified as new neurons by alternating between both filters and using a dual filter. Labeled neurons in NCM were counted in 10-12 sections from ~170 to 500 µm lateral to the midline of each hemisphere. The volume of NCM that was sampled was calculated by multiplying the section areas with the section thickness (6 µm). The overall neuronal densities were calculated by dividing the total number of double-labeled cells by total volume sampled (Pytte et al, 2010). None of the sections sampled were within 100 µm of an electrode track from electrophysiological recordings.

**Statistics**

For all of the statistical tests, the criterion for significance was set at p < 0.05. To see whether there were any differences in numbers of new neurons between the left and right hemispheres, we used the paired t-test, two-tailed. To determine relationships between new neuron numbers, song learning, and strength of auditory memory, we used regression analyses. We used Analysis of Variance (ANOVA) to see whether there was any difference in new neuron survival, and the neuronal responses to playback of songs between the statin-treated birds and control birds.
CHAPTER 3: LATERALIZATION OF NEW NEURONS IN NCM (AIM 1)

Songbirds store memories of the tutor song and other conspecific songs in NCM.

Electrophysiological recordings in NCM showed that neurons in the left hemisphere NCM had a lower firing rate than those in the right hemisphere NCM when the bird heard playbacks of familiar songs (Phan and Vicario 2010). This indicates that the neurons in the left NCM had a stronger memory strength for these songs than those in the right hemisphere. Hemispheric differences in NCM have also been demonstrated in gene expression and fMRI studies (Moorman et al., 2012; Voss et al., 2007).

New neurons are incorporated into NCM throughout the bird’s lifetime, and it has been established that numbers of new neurons in NCM are associated with social complexity (Lipkind et al., 2002; Adar et al., 2006; Barnea et al., 2008) and auditory experience (Pytte et al., 2010). In other systems, new neurons have been shown to play a role in learning and memory formation (Review: Leuner et al., 2006). However, it is not known whether the incorporation and survival of new neurons may differ between the left and right hemispheres. The first goal of this work is to determine whether the number of new neurons is lateralized in the zebra finch NCM, and to describe the relationship in numbers of new neurons between the left and right hemispheric NCM.

Specific Methods

Animals

Adult male zebra finches (n = 31) were 82 - 480 days old at the start of the experiment (mean = 213 days, SEM = 20.6). They were either tutored by their fathers (n = 27) or by a tape recorded adult song (n = 4). Father-tutored birds remained in family cages until after the critical period
for song learning, > 90 post-hatch day (phd), at which time they were housed in all male groups of 8-10 until the time of the experiment.

**BrdU injections**

All birds received intramuscular injections of bromodeoxyuridine (BrdU; 78 μl of a 10 mg/ml solution in 0.1 M tris buffered saline, TBS, pH = 7.4; Sigma) 3 times/day for 3 consecutive days to label mitotically active cells. Birds were perfused 28 +/- 2 days after the last BrdU injection to label 26 – 33 day old neurons.

**Quantification of new neurons**

To estimate the total number of new neurons added to NCM per day, based on our sampling counts, we used the Abercrombie correction equation (Guillery and Herrup, 1997). The mean diameter of the BrdU+ nuclei used in the equation was 6.56 μm, based on measured samples. We then divided the corrected new neuron density by 18, which was the number of hours that BrdU was made available for labeling from 3 injections in 3 days, to calculate the number of new neurons added in one hour. Then we multiplied this number by 24 to get an estimate of the true number of new neurons added in one day.

Abercrombie equation:

\[ N = n \left( \frac{T}{T+D} \right) \]

N is the total number of new neurons, n is the sampled number of new neurons counted, T is the thickness of the tissue, and D is the mean diameter of the nucleus.

**New neuron nuclear diameters**
Counting can be biased by the size of the nuclear diameter of the new neurons. In this way, differences we may find in numbers of new neurons between hemispheres could be explained by hemispheric differences in nuclear size. Nuclear diameter is known to decrease with neuron cell age (Kirn et al., 1991), therefore hemispheric differences in numbers of new neurons could reflect cell size differences due to hemispheric differences in the rates of new neuron maturation. To determine whether the new neurons differed in nuclear diameter between hemispheres, we measured and compared the longest diameter across the nuclei of a sample of BrdU-labeled neurons (n = 10 cells in each hemisphere for each bird, n = 21 birds). We used a 100 µm by 100 µm grid system to sample cells without selection bias, evenly distributed throughout 1-2 sections.

**Neuronal packing density**

Hemispheric differences in new neurons may reflect potential asymmetry in overall packing density of total numbers of neurons in NCM, and rates of neuronal turnover may be proportional to total neuron numbers in each hemisphere. In HVC, the incorporation of new neurons is linked to the loss of older, dying neurons (Alvarez-Buylla and Kirn, 1997). Therefore, it is possible that the same happens in NCM such that the hemisphere with more new neurons may have more existing neurons than the other hemisphere. The hemispheric asymmetry in the number of new neurons may also reflect hemispheric differences in the rate of neuronal turnover, among equivalent numbers of overall neurons. Therefore, the packing density of all NeuN+ neurons (including BrdU+ and BrdU– neurons) was measured in both hemispheres in a sample of 15 birds (chosen arbitrarily, blind to other data sets). For each bird, we used two NCM sections of each hemisphere matched for medial-lateral position across hemispheres. We counted all NeuN+ cells that had a visible nucleus in a 300 µm² box placed in the center of NCM.
Neuron Asymmetry Index

The Neuron Asymmetry Index (NAI) was used to calculate the relative difference in numbers of new neurons between both hemispheres within each bird. This difference was then normalized to the average number of new neurons across both hemispheres. A positive number indicated that the number of new neurons was lateralized to the left hemisphere (i.e. more in the left relative to the right). A negative number showed lateralization of new neurons to the right hemisphere.

\[ \text{NAI} = \frac{\text{LEFT neurons/mm}^2 - \text{RIGHT neurons/mm}^2}{(\text{L neurons} + \text{R neurons})/(\text{L area} + \text{R area})} \]

Calculation for the difference of the density of new neurons between the left NCM and the right NCM

In addition to the individual NAI calculations, we also calculated the difference in new neuron density across all birds as a group. The sum of the density of new neurons in the right NCM across all birds was subtracted from the sum of the density of new neurons in the left NCM across all birds. This difference, which was calculated as percentage, was then divided by the mean density of new neurons in the right and left NCMs across all birds.

Results

We found hemispheric asymmetry in the number of new neurons in NCM. New neuron density was significantly higher in the left NCM than in the right NCM (mean density per mm$^3$ +/- SEM, Left: 1368.65± 179.80; Right: 1041.00 ± 141.23; t (30) = 2.999, p = 0.005, two-tailed paired t-test, Figure 3A). 71% of the birds (22/31) in this sample had more new neurons in the left hemisphere (Figure 3B), and this difference is significant (p = 0.01, one-tailed sign test).
also found that within birds, the numbers of new neurons were correlated between the two hemispheres \(F (1, 29) = 52.56, R^2 = 0.64, p < 0.001\). The y intercept of 283.6 indicates a higher new neuron density in the left hemisphere across the population (Figure 4). An intercept of zero would indicate equal densities between hemispheres. Assuming that the new neurons were added to each hemisphere at a steady rate, based on our new neuron counts, we predicted that the left hemisphere would have approximately 1709 new neurons/mm\(^3\) per day whereas the right hemisphere would have 1307 new neurons/mm\(^3\) added per day, a difference of about 27\% (see methods for calculations).

Alternatively, this hemispheric difference in new neurons counted could be a reflection of hemispheric differences in the rate of neuronal maturation. The size of neuronal nuclei in HVC decreases as neurons mature and perhaps the same is true for NCM neurons (Kirn et al., 1991). Thus younger cells could be over-counted relative to older cells. Therefore, our data could reflect new neurons in the right hemisphere maturing faster (being overall older, smaller, and therefore underrepresented in our sampling).

Since the size of the cell and the size of the nucleus are positively correlated (Jorgensen, et al., 2007; Webster et al., 2009), measurements of the nucleus can reflect the size of the cell. We measured the longest diameter across the nuclei of the new neurons in a subset of birds, and found no difference in this measure between the left and right hemispheres (mean +/- SEM, Left: 6.66 +/- 0.18; Right: 6.46 +/- 0.22; t (13) = 0.91, p = 0.38, two-tailed paired t-test), suggesting that the new neurons in both hemispheres matured in the same rate.

We also wanted to determine whether hemispheric differences in new neurons reflect potential asymmetry in overall packing density of neurons in NCM, such that one hemisphere may have a larger overall population of neurons, which includes both new and existing neurons,
than the other hemisphere. Therefore, it is possible that the left hemisphere, which has more new neurons, may have fewer existing neurons than the right hemisphere. On the other hand, the left hemisphere could also have more existing neurons than the right hemisphere, which would mean that the left hemisphere NCM naturally has more neurons of all ages than the right hemisphere.

In a subset of birds, the neuron packing density, calculated as the density of NeuN+ cells did not differ between the left and right hemispheres (mean +/- SEM, Left: 1668.53 +/- 187.15; Right: 1784.65 +/- 179.22; t (14) = -0.94, p = 0.36, two-tailed paired t-test, Figure 5). NeuN is a protein expressed by mature neurons. Moreover, the areas of NCM sampled did not differ between the left and right hemispheres (mean +/- SEM, Left: 1.96 +/- 0.13; Right: 1.92 +/- 0.14; t (23) = 0.38, p = 0.71, two-tailed paired t-test), showing that the hemispheric differences in numbers of new neurons between the left and right hemispheres were not due to differences in the size of NCM sampled.

**Discussion**

We found the number of new neurons incorporated in NCM was lateralized with more new neurons in the left hemisphere (Figure 3). Most birds, but not all, had significantly more new neurons in the left hemisphere (Figure 4). Even though there were more new neurons incorporated in the left NCM, the volume of NCM and the packing density of all neurons (new + old) were not different between hemispheres. These results suggest that there was greater neuronal turnover in the left NCM than in the right NCM, and the right NCM had more mature neurons than the left NCM.

It would be interesting to see whether the neuronal density of proliferating and immature new neurons is also lateralized in the left hemisphere. If so, this could mean that the majority of the immature neurons was able to mature to become the neurons that we quantified. On the other
hand, if proliferation is equal across hemispheres or even lateralized to the right NCM, this could indicate that more young new neurons in the right NCM compared to left died prior to our quantification. Regardless, the dynamics underlying the resulting imbalance in left:right new neurons remains to be determined.
CHAPTER 4: NEW NEURONS IN NCM AND SONG LEARNING (AIM 2)

Zebra finches are “closed-ended” learners that learn their song only as juveniles and do not modify this song seasonally or in successive years. They learn their songs by imitating their tutor’s song, and can do so from memory without the tutor present during song learning (Konishi, 1965). Electrophysiological recordings have demonstrated that the left NCM has a stronger memory of the tutor’s song and other conspecific songs than the right NCM (Phan and Vicario, 2010), and the neuronal memory of the tutor’s song in the entire NCM is positively correlated with the success of song copying (Phan et al., 2006). Interestingly, the expression of immediate early genes (IEG) in response to playback of the tutor’s song is lateralized to the left hemisphere, but only in juvenile birds (Moorman et al., 2010; 2015). The lateralization of the expression of IEG was also directly correlated with the success of song copying (Moorman et al., 2015).

We found (Chapter 3), that the left NCM has more new neurons than the right NCM. We hypothesized that this hemispheric difference may correspond functionally to the lateralization of the tutor’s song memory shown by Moorman et al (2012; 2015). In other words, new neurons in the left hemisphere may play a role in the acquisition and/or storage of the tutor’s song memory. In songbirds, it was suggested that new NCM neurons contribute to learning and memory (Barnea et al., 2006; Adar et al., 2008), and we also know from a study in mice that the elimination of new hippocampal neurons impaired long-term memory (Synder et al., 2005), showing that new neurons play a role in memory formation and/or storage. If a similar relationship between new neurons and memory exists in birds, then birds with more new neurons in the left hemisphere may have stronger memory of the tutor’s song, which is directly linked to better song learning (Phan et al., 2006), than those birds with fewer new neurons in the left
hemisphere. Aim 3 will determine whether the numbers of new neurons in either or both hemispheres correlate with the quality of song copying. If so, this would suggest that left side lateralization of new neurons facilitates any of the multiple components of song learning including tutor song memorization.

**Specific Methods**

**Animals**

A subset of birds from Chapter 3 (n = 21) were used. Songs were recorded from each live-tutored bird (n = 18) at the start of individual isolation (~ day 25) and throughout song development until the day prior to neurophysiological recording at day 110. For individuals that were taped tutored (n = 3), songs were recorded from the end of song tutoring (approximately 85 phd, see general methods) until the day of neurophysiological recordings (day 110).

**Song Analysis**

Songs were recorded and song learning was calculated using software designed specifically for this assessment in zebra finches: Sound Analysis Pro 2011 (SAP, Tchernichovski, 2000). The adult, crystallized song of the birds (“learned song”) was compared with their tutor’s song (Samba or their father’s song) to assess accuracy of copying. A sample of 10 learned songs was compared with Samba (all songs are identical) or an exemplar of the father’s song. Sound Analysis Pro quantifies the spectral similarity of pairs of songs (in this case, learned song compared with the tutor’s song) using a sliding 10 ms frame across sound pairs based on the acoustic features: pitch, FM, AM, Wiener entropy, and goodness of pitch (SAP 2011 Online Manual). To produce an overall score of similarity, units for each feature are transformed to units of median absolute deviation from the mean. Three scores of song imitation were
produced. The “Percentage Similarity” is the percentage of the compared song files that pass a given threshold of similarity. “Accuracy” is a measure of the degree to which similar song segments are alike. “Sequence Match” is a measure of the degree to which notes or song elements appear in the same order across song pairs. The overall Similarity Index is a product of 2 components: Percentage Similarity and Accuracy (SAP 2011 Online Manual). The Similarity Index was compared with numbers of new neurons in NCM.

**Acoustic Parameters**

Pitch is a measure of sound frequency (high values have a short cycle and high frequency). Frequency modulation (FM) is the mean slope of changes in frequency over a given time. Amplitude modulation (AM) is a measure of the change in the amplitude envelope of sounds. Wiener entropy is a measure of the randomness of frequencies in any given time bin. For instance, white noise has a maximum Wiener entropy value of 1 and pure tones have a minimum value of zero. Goodness of pitch is a measure of the periodicity of harmonic structures in the song (e.g., modulated and non-modulated harmonic stacks have high values whereas noisy sounds and pure tones have low values) (Tchernichovski et al., 2000).

**Song Stereotypy**

Song stereotypy is a measurement of song maturation (Wilbrecht et al., 2002; Pytte et al., 2007). To assess stereotypy, we measured how similar each motif is to each other motif within a song bout of multiple motifs using SAP. A motif is a sequence of notes or elements separated by a silent interval that is longer than an inter-note interval (i.e. a “song”). In zebra finches, the acoustic structure and sequence of notes is highly stereotyped, therefore motifs within bouts are easily identified as repeating sequences of notes. For each bird, two bouts (one was from the
middle of the day; the other was from the end of the day) that had at least 6 motifs were chosen for analyses. For each bout, similarity between motifs was analyzed. First, adjacent motifs were compared with each other. Then the similarity score between one pair of adjacent motifs was compared with the similarity score between another pair of adjacent motifs as well as with the similarity score between motifs that were not next to each other (following methods of Pytte et al., 2007). Lastly, the motifs of the same bout positions were compared between the two bouts. The similarity scores from each comparison were averaged to obtain each bird’s overall stereotypy score (Wilbrecht et al., 2002; Pytte et al., 2007).

Statistics

We first performed Mann-Whitney U tests with SPSS to determine whether there were any differences between the live-tutored birds and the tape-tutored birds in the following: 1) Similarity Index; 2) numbers of new neurons in the left hemisphere, right hemisphere, and across both left and right hemispheres; 3) the degree of lateralization of the number of new neurons to the left hemisphere. Regression analyses were done to determine the correlations between the following variables: Similarity Index and the number of new neurons in the left hemisphere, right hemisphere, across both left and right hemispheres and NAI for all individuals (see Chapter 3 for calculations of neuron numbers and NAI). These analyses were conducted separately with siblings considered individually, sibling data averaged, live-tutored birds only, and tape-tutored birds only. Regression analyses were also done to determine the correlations between: 1) individual acoustic features (pitch, frequency modulation, amplitude modulation, Wiener entropy, and goodness of pitch) and the numbers of new neurons in the left hemisphere, right hemisphere, across both left and right hemispheres and NAI for all individuals. Finally, regression analyses were done to determine the correlations between song stereotypy and the
numbers of new neurons in the left hemisphere, right hemisphere, across both left and right 
hemispheres and NAI for all individuals. Significance levels were set for 0.05 for all analyses.

**Results**

**Overall relationship between song learning and the number of new neurons**

The success of song copying can be determined by the global measure of Similarity Index (SI), 
using Sound Analysis Pro (Tchernichovski et al., 2000). SI measures the similarity between the 
bird’s own song and the tutor’s song based on the match between 5 acoustic parameters: pitch, 
frequency modulation, amplitude modulation, Wiener entropy, and goodness of pitch (SAP 2011 
Online Manual). We did not find significant correlations between SI and the numbers of new 
neurons when we considered each hemisphere separately (Left hemisphere: F (1, 19) = 3.49, R^2 
= 0.16, p = 0.08, Figure 6A, Right hemisphere: F (1, 19) = 1.11, R^2 = 0.06, p = 0.30, Figure 6B) 
or when we combined both hemispheres (F (1, 19) = 2.49, p = 0.13, R^2 = 0.12, Figure 6C).

However, SI was significantly correlated with the relative difference in new neuron densities 
between the two hemispheres, which was calculated as a normalized “Neuron Asymmetry 
Index” (NAI, described in Chapter 3, Specific Methods) (F (1, 19) = 5.76, R^2 = 0.23, p = 0.027, 
Figure 6D). These correlations suggest that the quality of song copying may be functionally 
related to the *relative* number of new neurons in the left hemisphere compared with the right.

The SI is based on the component acoustic features. Therefore, we determined whether 
numbers of new neurons were also related to each feature independently. None of these song 
features (listed above) were correlated with numbers of new neurons in either or both 
hemisphere(s) or the NAI (p > 0.05 for all). These results suggest that the correlation between
NAI and SI was determined by a combination of song features and not driven by any particular acoustic parameter.

**Sibling**

Within this set of 21 birds, there were five sibling groups (n = 14 birds with at least one sibling). Previous work has shown that numbers of new neurons have a genetic component (in mice, Kempermann et al., 2006; and zebra finches, Hurley et al., 2008). Therefore, new neuron counts in brothers are not truly independent. To account for this, we redid the analysis after the values for brothers were averaged, with each mean brother-group considered as an individual data point. Similar to when each bird was considered individually, there was no correlation between SI and number of new neurons in either hemisphere (Left: F (1, 12) = 0.95, R² = 0.073, p = 0.349, Figure 7A; Right: F (1, 12) = 0.043, R² = 0.004, p = 0.840, Figure 7B). There was also no correlation when the numbers of new neurons in both hemispheres in this subset compared with SI (F (1, 12) = 0.44, R² = 0.04, p = 0.52, Figure 7C). As above, when the brothers were combined as a single value, SI and NAI were correlated (F (1, 12) = 10.51, R² = 0.47, p = 0.007, Figure 7D).

The birds in this analysis were either live-tutored or tape-tutored (see below). When we analyzed the data (averaged siblings) only with the live-tutored birds (n = 11), the results were consistent with the results of the full data set. The numbers of new neurons and the quality of song copying were not related when the hemispheres were considered either independently (Left: F (1, 9) = 0.06, R² = 0.007, p = 0.81; Right: F (1, 9) = 0.14, R² = 0.015, p = 0.72) or combined (F (1, 9) = 7.53 x 10⁻⁶, R² = 8.37 x 10⁻⁷, p = 1.0). SI and NAI were significantly correlated (F (1, 9) = 12.59, R² = 0.58, p = 0.006, Figure 7E).
In the small group of tape-tutored birds (n = 3), the quality of song copying was not related to the numbers of new neurons in the left hemisphere (F (1, 2) = 1.79, R² = 0.64, p = 0.41), in the right hemisphere (F (1, 2) = 17.69, R² = 0.95, p = 0.15), or both hemispheres combined (F (1, 2) = 31.95, R² = 0.97, p = 0.11). There was also no significant correlation between SI and NAI (F (1, 2) = 6.37, R² = 0.86, p = 0.24).

**Live-tutoring vs tape-tutoring: Song learning and new neuron numbers**

The birds used in this study (n = 21) included individuals that were tutored in home cages by their biological fathers (n = 18) as well as birds that were tutored in individual cages by recordings of a single adult male song played back to the juveniles (n = 3). It has previously been established that song copying is more accurate when a bird is trained with a live tutor rather than with a song playback from a speaker, presumably due to the social interaction (Deregnaucourt et al., 2013). We found that the live-tutored birds did have higher SI scores than the tape-tutored birds (U = 2.0, z = -2.51, p = 0.012). However, live-tutored birds did not have more new neurons than tape-tutored birds in the left hemisphere NCM, right hemisphere NCM, or the entire NCM (U = 13.0, z = -1.41, p < 0.05 for all). Moreover, the numbers of new neurons in the live-tutored birds were not more lateralized (to either side) than the tape-tutored birds (U = 19.0, z = -0.80, p < 0.05).

**Song Stereotypy**

Zebra finches learn to sing during a juvenile critical learning period, which ends around 90 post hatch days. After this period, the adult bird’s song is highly stereotyped (Wang et al., 2002). In the song motor pathway nucleus HVC, the number of new neurons decreases as the birds age in adulthood (Wang et al., 2002). Moreover, the number of new neurons in HVC is inversely
correlated with song stereotypy (Wilbrecht et al., 2006; Pytte et al., 2007). Therefore, in addition to the global score of SI, we also evaluated potential correlations between new neurons in NCM and song stereotypy (Wilbrecht et al., 2006; Pytte et al., 2007). The pathway that connects NCM and HVC is indirect (Foster and Bottjer, 1998). Therefore, we speculated that new neurons in NCM might also affect song stereotypy through their connections with HVC. However, we did not find a correlation between song stereotypy and new neurons in either or both hemisphere(s) of NCM, or the NAI (p > 0.05 for all). This suggests, that unlike in the song motor pathway, new neurons in NCM do not appear to be related to the quality of song production.

**Discussion**

**New neurons and song learning**

New neurons in the hippocampus are thought to play a role in learning (Snyder et al., 2005; Tashiro et al., 2007; Deng et al., 2009; Trouche et al., 2009; Aimone et al., 2011; Vukovic et al., 2013; Akers et al., 2014). NCM is critical for song learning and in particular stores a copy of the tutor song model that the bird attempts to imitate. Moreover, the quality of this model has been shown to be correlated with accuracy of song imitation (Phan et al., 2006). Therefore, we hypothesized that new neurons in NCM may contribute to song learning and expected to find a correlation between numbers of new neurons and the quality of song learning. However, our data did not support this idea: the absolute number of new neurons in either hemisphere (or both combined) was not correlated with the quality of song imitation. Instead, we were surprised to find 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 NCM, was positively correlated with song learning. Birds with more new neurons in the left NCM than the right NCM were able to
produce better song copies. The correlation between song learning and left-lateralization suggests not only a potential benefit for higher numbers of new neurons in left NCM, but also a disadvantage to having new neurons in the right hemisphere. If so, there may be a small optimal range of new neurons in right NCM, with a minimum number necessary for song learning, and a ceiling beyond which too many new neurons would interfere with developing or maintaining a song memory, or other processes underlying song learning.

**Lateralization of ZENK expression**

Lateralization of ZENK expression in response to song playback depends on the age of the bird and the song type that the bird is exposed to. In juvenile birds, there is more ZENK expression in the left NCM than the right hemisphere NCM in response to tutor’s song, suggesting that the memory of the tutor’s song is likely stored in the left hemisphere (Moorman et al., 2012; 2015). It is intriguing that the correlation we found between success in song learning and the left-side lateralization of new neurons in adults parallels the correlation reported by Moorman et al., (2012, 2015) between song learning and left-side lateralization of the expression of the immediate early gene ZENK in NCM in juveniles. However, unlike in the juvenile birds, Moorman et al. found no differences in the expression of ZENK between the left and right hemispheres in adult birds in response to tutor’s song (Moorman et al., 2012; 2015). This result could reflect the fact that the adult birds do not retain the tutor’s song memory, perhaps because it is not used as much as it is by the juvenile birds. Alternatively, memory storage may be retained but bilaterally represented in adults.

Interestingly, ZENK expression was lateralized to the left NCM in adult male zebra finches when the birds were exposed to conspecific songs (Avey et al., 2005). This is consistent with electrophysiology data showing stronger left NCM memory for conspecific songs than right
NCM memory (Phan and Vicario, 2010). These results suggest that left and right hemispheres process conspecific songs differently, and this may depend on the importance and relevance of the memory of a particular song type (i.e. tutor’s song vs. other conspecific songs). Currently, there are no studies directly comparing ZENK expression, electrophysiology, and the number of new neurons in any model system. But it would be interesting to compare new neurons with ZENK expression to determine whether young neurons preferentially express ZENK, and whether there are functional correlations between new neurons and ZENK. In this work, we found that the lateralization of the number of new neurons in the left NCM was directly related to the success of song learning, which is related to neuronal memory of the tutor’s song (Phan et al., 2006). Based on these findings, ZENK expression may be directly related to the relative number of new neurons between the two hemispheres in the adult birds.
CHAPTER 5: NEW NEURONS IN NCM AND MEMORY (AIM 3)

Aim 3 is to determine the relationship between the numbers of new neurons in either or both hemispheres and the strength of 20-hour memory storage of conspecific songs. In chapter 4, we reported that the lateralization of new neurons is correlated with the success of song learning, which is linked to the neuronal memory of the tutor’s song. Previous research showed that the left NCM had stronger 20-hour neuronal memory than the right NCM in response to conspecific songs (Phan and Vicario, 2010). This finding is similar to the lateralization of ZENK expression responding to conspecific songs in the adult birds (Avey et al., 2005). Since the left NCM has more new neurons than the right NCM, it is possible that there is a direct correspondence between the number of new neurons in the left NCM and 20-hour memory strength in the left NCM. In this Aim, we are testing this possibility.

Specific Methods

Animals

The same birds were used as in Chapters 3 and 4.

Electrophysiological Recording

The procedures are described in General Methods. In brief, electrophysiological recordings were made after song learning was complete, when the zebra finches were about 100-110 phd. At least 24 hours before recordings, electrodes were implanted over NCM bilaterally. Once the birds were recovered, they were put into isolated chambers for 20-hour memory testing.

20 Hour Memory Testing and Analysis
Birds in isolation were exposed to 200 repetitions of 8 novel conspecific songs (blocked, interstimulus interval = 8 seconds). After this exposure, the birds became familiar with these 8 songs, which were now called “familiar songs”. The following day, 20 hours later, neuronal responses were recorded to playback of 25 repetitions of these 8 familiar songs along with 8 additional novel songs in shuffled order. For assessment of auditory tuning properties at the recording sites, birds also heard sets of artificial sounds: pure tones and band-limited noise (0.5 – 5 kHz in 0.25 kHz steps; 5 repetitions, ISI 6 seconds). All stimuli were presented in free-field from a speaker located at a distance of 0.5 m directly in front of the subject at an average amplitude of 75 dB SPL (“A” scale).

Neuronal responses were calculated by subtracting the root-mean-square (RMS) voltage values of responses during playback (from stimulus onset to offset plus 100 ms) from the RMS of the control on each trial (500 ms immediately prior to stimulus onset). This calculation eliminated noise and baseline activity. To compute the RMS, each digitized value was squared, the mean of these squares over the response interval was computed, and the square root of that mean was taken. This provides a method of rectifying the multi-unit activity and computing its average power. The RMS responses on trials 2-6 were averaged to compute Absolute Response Magnitudes (ARMs) for each stimulus, as previously described (Phan and Vicario, 2010).

To measure memory strength of the familiar songs, we calculated the Relative Response Strength (RRS), which is the difference between the ARMs of the novel songs and familiar songs. When the neurons have a “memory” of the familiar song, the neuronal activity will be lower than when exposed to a novel song, i.e., the RRS will be positive.

\[
RRS = \frac{N_{ARM} - F_{ARM}}{(N_{ARM} + F_{ARM})/2}
\]
When RRS is zero, it means the responses to the playback of novel and familiar songs were equal, showing that the neurons have no “memory” of the familiar song. RRS value is first calculated across all of the recording sites in each hemisphere and then all of the values were averaged to produce a mean RRS value for each hemisphere per bird.

**Results**

Neuronal activity in NCM in response to song playbacks can be used to measure the strength of song memory since firing activity decreases as the bird becomes familiar with the song stimuli. (Phan et al., 2006). Here we used the Relative Response Strength (RRS), comparing NCM responsivity to familiar songs relative to novel songs, to measure the neuronal memory during playback of conspecific songs. An RRS of zero means that the neurons did not have a “memory” of the familiar song because there is no difference in neuronal activity in response to either song types. When the neurons fire less in response to a familiar song than to a novel song, it is considered that NCM has a “memory” of the familiar song, and this is represented by a positive number. A negative RRS indicates that the neurons fired more in response to the familiar stimulus than those responding to the novel song, which is assumed to indicate no memory for the familiar songs. Across all birds, both left and right hemispheres had a positive RRS, indicating a memory of the familiar song. However, the RRS was higher in the left hemisphere NCM than the right hemisphere NCM, suggesting a stronger memory in the left hemisphere than the right. This was the case when all siblings were considered individually (Left: 0.075 +/- 0.02 SEM; Right: 0.008 +/- 0.02 SEM; t (20) = 4.22, p < 0.001, two-tailed paired t-test, Figure 8A), and when the values for brothers were averaged (see Ch. 4 for information about siblings) (Left:
Although the function of new neurons in NCM has not been established, it has been suggested that new neurons in NCM contribute to learning and memory (Barnea et al., 2006; Adar et al., 2008.) Therefore, we predicted a positive relationship between the numbers of new neurons in NCM and the memory strength of the song stimuli. However, we did not find this result. The left hemisphere RRS was not correlated with the number of new neurons in the left NCM -- either when all birds were considered (F (1, 18) = 1.36, R$^2$ = 0.07, p = 0.26, Figure 9A), or when the brothers were combined (F (1, 12) = 0.57, R$^2$ = 0.05, p = 0.46, Figure 9B).

Likewise, the right hemisphere RRS was not correlated with the number of new neurons in the right NCM (Individual birds: F (1, 18) = 0.001, R$^2$ = 4.4 x 10$^{-5}$, p = 0.98; Combined brothers: F (1, 12) = 0.02, R$^2$ = 0.002, p = 0.89, Figures 9C and 9D).

We then assessed whether there was a relationship between the degree to which the number of new neurons is lateralized and memory strength using RRS. Even though RRS was higher in the left hemisphere, RRS in the left hemisphere was not correlated with the Neuron Asymmetry Index (Individual birds: F (1, 18) = 0.73, R$^2$ = 0.04, p = 0.40; Combined brothers: F (1, 12) = 0.74, R$^2$ = 0.06, p = 0.41, Figures 10A and 10B). Similarly, when the birds were considered individually, RRS in the right hemisphere was not correlated with the lateralization index (F (1, 18) = 2.8, R$^2$ = 0.14, p = 0.11, Figure 10C). However, there was a correlation between RRS in the right hemisphere and lateralization of new neurons when the brothers were combined (F (1, 12) = 5.45, R$^2$ = 0.31, p = 0.038, Figure 10D). Interestingly, this shows that when there were more new neurons in the left hemisphere relative to the right, there was a
stronger 20-hour memory of the conspecific songs in the right hemisphere than in the left hemisphere

**Discussion**

*New neurons and memory of conspecific songs*

Our results showed that both left and right hemispheres contained memories of familiar songs heard 20 hours earlier, and that the left NCM had stronger memories than the right NCM. This hemispheric difference in memory storage may be a reflection of the lateralization of activation to the left hemisphere midbrain (MLD), left Field L and left Area X in response to the playback of familiar conspecific songs as shown by fMRI (Poirier et al., 2009).

However, it is puzzling that the degree of left side lateralization of new neurons was positively correlated with the weaker memory in the right hemisphere instead of the stronger memory in the left hemisphere. Perhaps, this can be explained if the left hemisphere and right hemisphere process or store different aspects of the song. For example, the left side may store memories of song components or features while the right stores more global aspects of songs. This hemisphere difference in information processing is seen in both avian (Yamazaki et al., 2007) and human visual systems (see review Hellige, 1996). In humans, it was found that with split brain patients and people with both hemispheres intact, the right hemisphere processed the global aspects of a visual stimulus and the left hemisphere processed the same visual stimulus in a local scale (see review Hellige, 1996.)

Another possible explanation for this correlation is that the new neurons have a negative effect on memory. A recent study suggests that new neurons may interfere with the storage of old hippocampal dependent memories (Akers et al., 2014). Therefore, if the new neurons in the
right NCM interfere with the memory stored in the right side, then having fewer new neurons in the right NCM would correspond to a better memory in the right NCM. It was reported that new neurons were necessary for learning and the retention of long-term memory in the mouse hippocampus (Snyder et al., 2005; Deng et al., 2009). If this is the case in NCM, more new neurons in the left hemisphere would be positively correlated with memory strength. However, we did not find a correlation between the memory strength in the left NCM and the lateralization of new neurons or absolute number of new neurons in the left hemisphere, suggesting that this model does not apply to NCM.

In the left hemisphere, the strong memory strength in response to the song playbacks may be related to the idea of an “ensemble”, which is formed by the interactions between neurons that respond to a specific acoustic feature (Chew et al., 1995). We found that the left hemisphere had stronger relative response strength compared to the right hemisphere. It is possible that this difference is due to the difference in the structure of an ensemble. The neurons in the left NCM may form multiple interactions to respond to different acoustic features, and the combination of the responses from each interaction would result in a strong memory strength. At the same time, the ensembles in the left NCM are probably composed of new neurons, which have properties contributing to plasticity, and are important to the retention of memory (Synder et al., 2001; Schmidt-Hieber et al., 2004). However, the right NCM may have fewer ensembles that tune to individual features and more that tune to the global song structure. Since the right NCM had fewer new neurons than the left NCM, the ensembles in the right NCM, which are mostly composed of older neurons, may be weaker than those in the left NCM. Thus, the right NCM, which tunes to global structure, had a lower overall memory strength.
CHAPTER 6: EFFECTS OF ATORVASTATIN ON LEARNING, MEMORY AND NEW NEURONS IN JUVENILE ZEBRA FINCHES (AIM 4)

Statins lower cholesterol production in the liver by inhibiting the rate limiting enzyme HMG-CoA, and consequently blocking the downstream mevalonate pathway. Statins that can cross the blood-brain barrier have been shown to affect brain cholesterol (Locatelli et al., 2002; Kirsch et al., 2003; Burns et al., 2006), which is important for neuronal development (Review: Zhang and Liu, 2015). One of the most commonly prescribed statins is atorvastatin (Lipitor), which has intermediate lipophilicity and can cross the blood-brain barrier. While some statin users have reported problems with memory (Wagstaff et al., 2003), other researchers have found no relationship between statins and cognitive impairments in healthy individuals (Ott et al., 2015), in people with mild cognitive impairments (Bettermann et al., 2012), and in people with Alzheimer’s disease (Ott et al., 2015). However, due to the potential effects of statins on cognition, in 2012, the FDA required all statins to carry a warning label advising users about the possible risks (Sahebzamani et al., 2014). Even though it is still unclear whether statins affect learning and memory in adults, the FDA has approved 4 kinds of statins to children as young as 8 years old with familial hypercholesterolemia (Stein, 2007). Currently, the effectiveness of statins to treat familial hypercholesterolemia in children is mostly extrapolated from the results of studies of adults (O’Gorman et al., 2011). Moreover, research on statins’ effects on children’s cognitive development is limited to a single study of their school performance over a course of 2 years (Wiegman et al., 2004). The goal of this Aim is to determine the effects of atorvastatin on learning, memory and new neurons in birds that were treated during the critical learning period. This Aim will focus on two neuronal populations and 2 regions: immature neurons (1-3 weeks old) and mature new neurons (30 days old) in HVC of the motor pathway, and 30 day old
neurons in NCM. Results from this work may clarify the potential effects of statins on learning, memory, and neural substrates in developing juvenile brains.

**Specific Methods**

**Experimental overview**

In this Aim, we investigated the long term effects of statins on learning, memory and neurogenesis in zebra finches. During the entire experiment, the birds were isolated in individual chambers. Juvenile birds were orally administered either with a clinical dosage of atorvastatin or water vehicle within their critical learning period starting at around 45 days old for about 50 days. They were trained with the tutor’s song (Samba) from 45 days old to 80 days old. When the birds were around 110 days old, the birds’ own songs were recorded to assess the quality of song imitation. Prior to song recordings, a recording chamber and a head fixation pin were cemented over the recording region. After recovering from the surgery (24 – 48 hours post craniotomy), electrodes were advanced bilaterally into NCM (as in Phan et al., 2006; Phan and Vicario, 2010). Then the birds were presented with songs of novel conspecifics (8 songs, 1 motif each). The following day (20 hours later), NCM responses to playback of the tutor’s song, bird’s own song, and the conspecifics songs that were presented previously and new conspecific songs were obtained in order to test the strength of memory for the tutor song and 20-hour memory of conspecific songs. One – three days after recordings, the birds were sacrificed and brains were processed for immunohistochemistry. In this work, we focused on the numbers of 30-day old new neurons in NCM and HVC. In HVC, we also determined the effects of statin treatments on the number of immature (1-3 weeks old) new neurons and the morphologies of all neurons, which included neurons that were 30-days old, and neurons that were not birthdated.
Animals

Juvenile birds (n = 17) were 18 – 49 days old (mean = 37.4 days, SEM = 2.26) at the start of the experiment. When the birds were 10 days old, their fathers were removed from their cages to prevent exposure to an adult song (following methods of Phan et al., 2006). They were housed with their mothers and siblings until they reached 25 days old at which time they can feed themselves. At 25 days old, each juvenile bird was moved to an individual sound attenuated chamber. Birds were exposed to a tutor song (Samba) from a tape recording between the ages of 45 days old to 80 days old (Figure 4, see General Methods for tape-tutoring). Throughout the experiment, birds were given a high-cholesterol diet (see below for details). When the birds were 110 days old, the birds’ own songs were recorded. Electrophysiological recordings in NCM were also performed on the same day (Figure 11). The birds were sacrificed on the following day and brains were processed for immunohistochemistry (see General Methods for histology and immunohistochemistry).

Statin administration

The juvenile male zebra finches were 18 – 49 days old (mean = 37.4 days, SEM = 2.26) at the start of statin administration.

Birds were divided into two groups. Birds were either orally administered 40 mg/kg atorvastatin (Lipitor®, obtained from Pfizer) in 50 μl water (n = 9) or given 50 μl water as vehicle once daily (n = 8). The mean number of days of statin or water administration was 52.5 (49 - 56) days. (Figure 11).

Song analysis
Individual acoustic features (amplitude, pitch, principal contour, mean frequency, goodness of pitch, frequency modulation, amplitude modulation, Wiener entropy, continuity over time, continuity over frequency and duration of state) were compared between birds treated with statins and those not treated with statins to examine potential differences in song structure. In addition, to specifically assess the quality of song copying, we compared the birds’ songs with their tutor’s song (Samba) using the Similarity Index, Accuracy scores, Sequence scores, and measures of separate acoustic features (pitch, frequency modulation, amplitude modulation, Wiener entropy, and goodness of pitch). Accuracy scores measure the similarities of the individual sections between the birds’ own songs and the tutor’s song. Sequence scores measures the similarity of the sequence of the syllables produced between the birds’ own songs and the tutor’s song.

**Memory of the tutor’s song**

Memory strength of the tutor’s song was measured with the familiarity index (FI) and the (RRS). The (FI) is based on the adaptation rate of the neuronal population in response to successive song playbacks (described in the Introduction, Chapter 1). The adaptation rate is the slope of the decreasing magnitude of responses of NCM to song playbacks. The FI for the tutor’s song is the ratio of the adaptation rate to the playback of a novel song and the adaptation rate to the playback of the tutor's song. An FI value that is equal to 1 means that the adaptation rates for both types of songs were the same, and this is interpreted as a lack of memory of the tutor's song. When the FI is greater than 1, the adaptation rate of the novel song is higher than that of the tutor's song, indicating a memory of the tutor's song.

The Relative Response Strength (RRS) is the difference in the Absolute Response Magnitudes (ARMs) between the novel songs and familiar songs. This difference is normalized
by the means. When the neurons have a “memory” of the familiar song, the neuronal activity will be lower than when exposed to a novel song, i.e., the RRS will be positive. When RRS is zero, it means the responses to the playback of novel and familiar songs were equal, showing the neurons have no “memory” of the familiar song.

$$\text{RRS} = \frac{N_{\text{ARM}} - F_{\text{ARM}}}{(N_{\text{ARM}} + F_{\text{ARM}})/2}$$

Recording values are the average of values from both hemispheres.

**20-hour song memory**

To prepare for recording, animals were anesthetized, placed in a stereotaxic device, and was outfitted with a metal head post which was attached to the skull with dental cement (Dentsply Caulk, Milford DE). To prepare for electrophysiological recording, during the same surgery, the outer layer of skull was opened over the target area, and dental cement (Dentsply Caulk, Milford, DE) was used to form a chamber for chronic recording. The head post and a custom-made body tube permitted comfortable immobilization of awake animals during testing. Once the birds recovered (36 – 48 hours later), each individual was put into an isolated chamber for 20-hour memory training and testing. Birds were exposed to 200 repetitions of 4-8 conspecific songs (blocked, ISI 8 seconds) for training. After this training, these songs were putatively now “familiar” songs. The following day, 20 hours after training, birds, which were restrained and awake, were then exposed to a playback of 25 repetitions of the 4 familiar songs along with 4 novel conspecific songs in a shuffled order in an isolated chamber. A multielectrode microdrive (Thomas Recording, Giessen, Germany) controlled the depth of 16 quartz-platinum/tungsten microelectrodes, allowing for simultaneous recording at multiple sites (8 in the left hemisphere, 8 in the right). Neuronal responses to song playbacks from both
hemispheres were averaged for FI and RRS calculations (see Chapter 5 Specific Methods for details on 20-hour memory testing and analysis).

**Immunohistochemistry**

We only used the left hemisphere because there was no a priori reason to suspect that the drugs might affect the two hemispheres differently.

*Doublecortin*

Doublecortin (DCX) is expressed in immature new neurons (1-3 weeks old). Processing methods are described in General Methods.

*BrdU/NeuN*

Labeled with BrdU and NeuN was used to identify 30-day old new neurons; described in General Methods.

**Cell qualification**

See General Methods for mapping NCM and HVC. We only used the left hemisphere reasoning that statin drugs likely affect the entire system. There is no evidence that the drugs affect the two hemispheres differently. DCX-expressing neurons were identified by DAB labeling using bright field microscopy. DCX+ cells were marked and cells per area HVC sampled were computed. BrdU-labeled new neurons were identified by co-expression of BrdU and NeuN which are labeled with a green fluorescent marker and a red fluorescent marker respectively.

**Cell morphology**

Contours of the cell body of all neurons, which included neurons that were 30-day old, and neurons that were older than 30-day old, were traced with dark field optics using Neurolucida
(MicroBrightfield). Neurolucida automatically computed soma contours using the following measurements.

1. **Aspect Ratio**: measures the degree of contour flatness. It is the ratio between the longest diameter (Feret Max) and the shortest diameter (Feret Min). Values closer to 0 indicate a flat contour; values closer to 1 indicate a round contour.

   $$ Aspect Ratio = \frac{Maximum Diameter}{Minimum Diameter} $$

2. **Roundness**: measures the degree of contour flatness, using a different equation from that of aspect ratio. It is the square of an object's compactness. As with aspect ratio, values closer to 0 indicate a flat contour; values closer to 1 indicate a round contour.

   $$ Roundness = \frac{4 \times Area}{\pi \times Feret Max^2} $$

3. **Compactness**: measures the relationship between the area and maximum diameter. Values closer to 1.0 indicate a circle; values closer to 0.8 indicate a square.

   $$ Compactness = \sqrt{\left(\frac{4}{\pi}\right) \frac{Area}{Feret Max}} $$

4. **Form Factor**: measures the complexity of the perimeter. Values closer to 0 indicate a rough and jagged perimeter; values closer to 1 indicate a smooth perimeter.

   $$ Form Factor = \frac{4\pi Area}{Perimeter^2} $$

5. **Shape Factor**: measures contour complexity. Values that are closer to 3.5 indicate a circular contour; values greater than 3.5 indicate a convoluted contour.

   $$ Shape Factor = \frac{Perimeter}{\sqrt{Area}} $$

6. **Convexity**: measures contour convexity. An object without indentations has a convexity value of 1. Concave objects have convexity values less than 1.
Convexity = \frac{Convex Perimeter}{Perimeter}

Statistics

We used the two-tailed t-test assuming equal variance to determine whether there were differences between the statin-treated birds and the control birds in the following: 1. Numbers of new (30 day old) neurons in NCM; 2. Numbers of new (1-3 weeks old) neurons in HVC; 3. Neuronal morphological measurements; 4. Song learning: Similarity Index, Percentage Similarity, Accuracy score, Sequence score; 5. Song learning: Acoustic parameters that the Similarity Index was based on (Wiener entropy, frequency modulation, pitch, goodness of pitch, and amplitude modulation); 6. 20-hour neuronal memory of novel conspecific songs.

The non-parametric Mood’s median test was used to determine whether there was a difference between control and statin-treated birds in the memory strength of the tutor’s song.

For all of the tests, the alpha level was set at 0.05 except for the following:

1) The repeated comparisons of the acoustic parameters in which the alpha level was corrected by Bonferroni correction to be 0.0063.

2) The tests of morphological features of 30-day neurons and the tests of sizes of older neurons were corrected using the Benjamini and Hochberg false discovery rate procedure (Benjamini and Hochberg, 1995). This is one of the least stringent of the correction methods and was chosen because we wanted to minimize the chances of Type 2 errors. The significance levels calculated by this method are 0.04 and 0.05.

Results

Song comparisons between statin-treated birds and controls
No differences were found between the song features of the statin-treated birds’ songs and those of the control birds’ songs (amplitude, pitch, principal contour, mean frequency, goodness of pitch, frequency modulation, amplitude modulation, Wiener entropy, continuity over time, continuity over frequency and duration of state, p > 0.05, Figure 12).

**Song learning in statin-treated birds and controls**

Treating birds with 40 mg/kg of atorvastatin did not affect the quality of song copying based on the global Similarity Index (means +/- SEMs, Control birds: 68.5 +/- 5.38; Statin-treated birds: 54 +/- 4.76; t (13) = 2.03, p = 0.067, two-tailed t-test, Figure 13). The Accuracy scores were also not different between the control birds and the statin-treated birds (means +/- SEMs, Control birds: 71.07 +/- 0.62; Statin-treated birds: 70.98 +/- 0.74; t (8) = 0.09, p > 0.05, two-tailed t-test). The Similarity in Sequences between the birds’ own songs and the tutor’s song likewise did not differ between control and statin-treated groups (Means +/- SEMs, Control birds: 49.47 +/- 5.41 SEM; Statin-treated birds: 51.63 +/- 5.94 SEM; t (11) = -0.25, p > 0.05, two-tailed t-test).

Next, we compared the 5 acoustic parameters (pitch, frequency modulation, amplitude modulation, Wiener entropy, and goodness of pitch), which comprise the global SI, between the tutor’s song (Samba) and the birds’ own songs. There were no differences between the songs in any of the 5 parameters (Table 2 and Figure 14).

**Effects of atorvastatin on neuronal memory of the tutor’s song**

Next, we assessed whether atorvastatin affected the neuronal memory of the tutor’s song, measured by the neuronal responses to song playbacks. When a bird is exposed to a novel song, the adaptation rate (or decrease in response rate) is a high value, indicating that the neurons do not have a memory of the novel song. If a bird is exposed to a familiar song, the adaptation rate
is smaller due to relatively low magnitude of neuronal responses. As expected, we found that the control birds had a higher adaptation rate to novel songs than to tutors songs. Statin-treated birds had less of a difference between adaptation to the novel songs and the tutor’s songs. Using the familiarity index (FI) to compare the adaptation rates in response to novel songs and the tutor’s song, we found that the control birds had a greater relative difference in adaptation rates, indicating a stronger neuronal memory of the tutor’s song than the statin-treated birds (Control birds: n = 7, median = 1.36; Statin-treated birds: n = 6, median = 1.08; \( \chi^2 = 3.90, p = 0.048 \), two-tailed Mood’s median test, Figure 15). However, when we used the Relative Response Strength (RRS) to measure the memory strength of the tutor’s song, we found no difference in RRS values between the control and statin-treated birds (Control birds, n = 7, median = -0.001; Statin-treated birds, n = 7, median = -0.011; \( \chi^2 = 0.286, p = 0.593 \), two-tailed Mood’s median test).

**Relationship between SI and FI**

The success of song copying, measured with SI, is related to whether the bird had the memory of the tutor’s song, measured with FI. Birds that had copied the tutor’s song well (had a high SI value) also had a better memory of the tutor’s song (high FI) (Phan et al., 2006). However, we did not find a significant relationship between SI and FI in control birds, which was inconsistent with the previous finding (Phan et al., 2006). This relationship was also not significant in statin-treated birds. These results could be due to the low sample sizes in both groups. Nevertheless, the correlation between these two variables (SI and FI) was higher in the control birds than in the statin-treated birds, suggesting that perhaps statin treatment disrupted either or both SI and FI, although not significantly (Control birds: \( F (1, 5) = 1.45, R^2 = 0.225, p = 0.282 \); Statin-treated birds: \( F (1, 4) = 0.04, R^2 = 0.01, p = 0.852 \), Figure 16).


**Effects of atorvastatin on 20-hour neuronal memory of conspecific songs**

Even though atorvastatin affected tutor’s song memory as described above, treatments did not have an effect on 20-hour memory of conspecific songs as measured with FI (mean +/- SEM, Control birds, 1.158 +/- 0.030; Statin-treated birds, 1.243 +/- 0.054; t (14) = 1.372, p = 0.191, two-tailed t-test, Figure 17A), and RRS (mean +/- SEM, Control birds, 0.0657 +/- 0.02; Statin-treated birds, 0.0637 +/- 0.0304; t (14) = -0.056, p = 0.957, two-tailed t-test, Figure 17B).

**Effect of atorvastatin on the number of new neurons in NCM and HVC**

New neurons play a role in memory formation and retention (Tashiro et al., 2007; Trouche et al., 2009; Goodman et al., 2010; Aimone et al., 2011; Akers et al., 2014). Treating cultured neurons with statins increased cell death (Michikawa and Yanagisawa, 1999; Pavlov et al., 1995), therefore, it is possible that oral administration of atorvastatin in zebra finches would affect neuronal survival in NCM, which would lead to the impairment of neuronal memory of the tutor’s song. Contrary to this idea, we found that atorvastatin did not affect the number of 30-day old new neurons in NCM (mean (number per mm²) +/- SEM, Control birds: n = 7, 8.68 +/- 2.18; Statin-treated birds: n = 5, 9.88 +/- 4.23; t (10) = -0.27, p = 0.78, two-tailed, Figure 18A).

Another factor that can affect the Similarity Index is whether a bird has the ability to learn to produce his song. If a bird has a sufficient memory of the tutor’s song but was not able to learn to produce a copy, the bird’s own song may not be similar to the tutor’s song. New neurons in HVC of the motor pathway, are important for song learning, specifically for song stability (Pytte et al., 2012). Moreover, it was shown that the number of neurons in HVC responding to the bird’s own song is positively correlated with the quality of song copying (Bolhuis et al., 2012). It is thought that a memory of the bird’s own song during development is used to compare with the memory of the tutor’s song in order to produce a good song copy. If
new neurons in HVC contribute to the memory of the bird’s own song, the trend towards a lower similarity score in statin-treated birds might have resulted from an effect of statins on number of the new neurons in HVC. However, we found that atorvastatin treatments did not affect the numbers of 30-day old new neurons in HVC (mean number per mm$^2$ +/- SEM, Control birds: n = 7, 12.07 +/- 1.13; Statin-treated birds, n = 6, 13.08 +/- 1.30; t (11) = -0.588, p = 0.569, two-tailed t-test, Figure 18B). The number of immature neurons that were 1-3 weeks old, which expressed doublecortin, was also not different between the control birds and the statin-treated birds (mean number per mm$^2$ +/- SEM, Control birds, n = 7, 246.98 +/- 29.12; Statin-treated birds: n = 6, 238.34 +/- 43.98; t (11) = 0.17, p = 0.87, two-tailed t-test, Figure 18C). Interestingly, even though the number of doublecortin-expressing neurons was not correlated with SI values in control birds (F (1, 5) = 4.36, R$^2$ = 0.466, p = 0.091, Figure 19A), we found a trend towards a significant positive correlation in statin-treated birds (F (1, 4) = 6.944, R$^2$ = 0.635, p = 0.058, Figure 19A). The numbers of 30-day old new neurons were not correlated with SI values in either control birds or statin-treated birds (mean +/- SEM, Control birds: F (1, 5) = 2.163, R$^2$ = 0.302, p = 0.201; Statin-treated birds: F (1, 4) = 0.0015, R$^2$ = 0.0004, p = 0.971, Figure 19B). Perhaps, this suggests that the immature neurons were more involved in the process of song copying in statin-treated birds.

**Effects of atorvastatin on the morphology of HVC neurons**

In the second part of this work with HVC, we investigated whether statin treatments affected the morphology of HVC neurons. *In vitro* studies demonstrated that statin-treated neurons had damaged organelles, fragmented neurites (Pavlov et al., 1995; Michikawa and Yanagisawa, 1999) and compacted nuclei with condensed chromatin (Pavlov et al.; 1995; Tanaka et al., 2000). Although we found that atorvastatin did not affect the numbers of new neurons, we hypothesized
that statin treatments could damage the neuronal membrane, which is composed of lipids and cholesterol, and could subsequently affect the integrity of the neurons and alter their shape.

We found that 30-day old neurons in statin-treated birds (n = 6) were flatter than those in the control birds (n = 8) as measured by aspect ratio (mean +/- SEM, Control birds: 0.697 +/- 0.011; Statin-treated birds: 0.654 +/- 0.014; t (12) = -2.437, p = 0.031, two-tailed t-test, Figure 20A), and roundness (mean +/- SEM, Control birds: 0.495 +/- 0.015; Statin-treated birds: 0.440 +/- 0.018; t (12) = -2.368, p = 0.036, two-tailed t-test, Figure 20B). For both aspect ratio and roundness, circular objects have values that are close to 1. We also looked at the compactness of the neurons, which is related to roundness, and found that there was a trend towards a significant difference such that neurons in the statin-treated birds were less compact than those in the control birds (mean +/- SEM, Control birds: 0.537 +/- 0.015; Statin-treated birds: 0.492 +/- 0.020; t (12) = -1.84, p = 0.086, two-tailed t-test, Figure 20C).

Atorvastatin administrations affected both contour complexity and perimeter complexity. Contours of 30-day old neurons in statin-treated birds were more convoluted than those in control birds as measured by shape factor, in which a value greater than 3.5 indicates a convoluted contour (mean +/- SEM, Control birds: 4.07 +/- 0.042; Statin-treated birds: 4.248 +/- 0.05; t (12) = 2.75, p = 0.018, two-tailed t-test, Figure 20D). Moreover, the new neurons in the statin-treated birds had a rougher and more jagged membrane than those in control birds as indicated by low form factor values (mean +/- SEM, Control birds: 0.77 +/- 0.014; Statin-treated birds: 0.718 +/- 0.015; t (12) = -2.523, p = 0.027, two-tailed t-test, Figure 20E). Despite the morphological differences, the sizes of these neurons in statin-treated birds were not different from those in control birds (mean +/- SEM, Control birds: 57.012 +/- 5.209; Statin-treated birds: 53.177 +/- 2.927; t (12) = -0.584, p = 0.57, two-tailed t-test, Figure 20F).
To determine whether statin treatments affected the rest of the neuronal population, we traced the contours of neurons that did not contain BrdU. Presumably, most of these cells were older than 30-days old. Most new neurons do not survive in the first weeks of birth in songbirds (Alvarez-Buylla and Nottebohm, 1988). Those that do, survive for many years (Walton et al., 2012). Therefore, we reasoned that the majority of non BrdU-labeled neurons that we quantified were likely older than the 30 day old BrdU-labeled cohort. Interestingly, unlike the 30-day old neurons, atorvastatin treatments did not affect the morphology of the older neurons (p > 0.05 for all contour measurements) but had an effect on the neuronal sizes. Overall, the mean area of the somas of the older neurons in statin-treated birds was smaller than that of control birds (mean +/- SEM, Control birds: 81.48 +/- 3.621; Statin-treated birds: 66.49 +/- 3.842; t (11) = -2.836, p = 0.016, two-tailed t-test, Figure 21A). Predictably, the maximum and minimum diameters of these neurons in statin-treated birds were also smaller than those in control birds (mean +/- SEM, Feret Max: Control birds: 13.919 +/- 0.228; Statin-treated birds: 12.678 +/- 0.474; t (11) = -2.476, p = 0.031, two-tailed t-test, Figure, 21B; Feret Min: Control birds: 7.774 +/- 0.201; Statin-treated birds: 6.995 +/- 0.107; t (11) = -3.251, p = 0.0077, two-tailed t-test, Figure 21C).

**Discussion**

We found that when zebra finches were treated with atorvastatin during the critical learning period for song learning, there was a trend towards a significant impairment of song copying, measured with a Similarity Index using Sound Analysis Pro (Tchernichovsky et al., 2000). Statin treatments did not affect copying of the 5 component parameters (pitch, goodness of pitch, frequency modulation, amplitude modulation and Wiener entropy), that comprise the Similarity Index. Overall, there were no measureable differences in acoustic structure between the statin-treated birds’ songs and the control birds’ songs. Interestingly, statin treatments impaired the
neuronal memory of the tutor’s song, which was measured by the familiarity index (FI). Even though the statin-treated birds had weaker neuronal memories of the tutor’s song than the control birds, 20-hour neuronal memories of other conspecific songs were not affected by the treatments. Unexpectedly, however, statin treatments did not affect the number of new neurons in NCM, which was related to the quality of song learning (Chapter 4). It is possible that the trend towards a significant impairment of song copying was due to the bird’s inability to learn to produce the song, which we speculated may involve new neurons in HVC (rather than NCM). However, we found that statin treatments did not affect the number of 30-day old neurons or 1-3 week old neurons in HVC. Instead, we found that statin treatments affected the morphology of 30-day old new neurons and the sizes of the neurons that were putatively older than 30-day neurons. These results are the first to illustrate effects of statins on neurons *in vivo*.

**Statins and memory**

The memory of the tutor’s song is stored in NCM (Phan et al., 2006; Bolhuis et al., 2000), and is retrieved during song learning (Solis and Doupe, 2000). We found that there is a significant effect of statins on the birds’ neuronal memory of the tutor’s song. Since we know from rodent studies that adult-born new neurons are important for the storage of memories (Synder et al., 2005; Tashiro et al., 2007; Deng et al., 2009), we initially hypothesized that the effects of statins on neuronal memory would be an indication of the effects of statins on the number of new neurons in NCM. However, contrary to our hypothesis, the number of new neurons in the left NCM was not affected by the statin treatments. Our laterализation work demonstrated that the quality of song copying is positively correlated with the laterализation of the numbers of new neurons in the left NCM (Chapter 4 and Tsoi et al., 2014). However, since there is no a priori knowledge that statins affect the left and right hemisphere differently, it is unlikely that statins...
would affect the lateralization of new neurons in NCM especially when statins did not affect the number of new neurons in the left hemisphere. However, this remains a possibility.

It is also possible that statins affected the axons and dendrites of the neurons in NCM and consequently impaired the synaptic connection between neurons. Treating cultured hippocampal neurons with statins reduced the number of lipid rafts located in the dendrites as well as the overall synapse density (Hering et al., 2003). The reduction of neuronal memory of the tutor’s song in statin-treated birds might be an indication of the reduction of the number of synapses in NCM. It is surprising that statins affected only the neuronal memory of the tutor’s song and not the memory of the conspecific song.

**Statins and neuronal morphology**

Atorvastatin, which we used in this work, has an intermediate lipophilicity, and can cross the blood-brain barrier based solely on its lipophilic property (Shobab et al., 2005). Statins that can cross the blood-brain barrier can decrease brain cholesterol (Locatelli et al., 2002; Kirsch et al., 2003; Burns et al., 2006). Therefore, we proposed that atorvastatin treatments may affect the plasma membrane of the neurons, which is composed of lipids and cholesterol. *In vitro* work has shown that statins affected the microdomains within the plasma membrane called lipid rafts, which are also composed of cholesterol and sphingolipids (Hering et al., 2003). These microdomains are important for cell signaling, trafficking of membranes and proteins, as well as the regulation of actin cytoskeleton (Hering et al., 2003). Therefore, statin treatments may impair the cytoskeletal structure of the cell body. Treating cultured neurons with mevastatin (compactin) reduced microtubule assembly by affecting phosphorylation of the microtubule-associated protein 2 (MAP2) (Fan et al., 2002). Our results showed that 30-day old new neurons in statin-treated birds were flatter, less compact, more convoluted and had rougher perimeters.
than those in control birds. These morphological changes could reflect the effects of statins on the integrity of the plasma membrane as well as the cytoskeletal structure in the cell body. *In vitro* reduction of neuronal cholesterol caused the membranes to become less rigid (Sooksawate and Simmonds, 2001). It is possible that, in this current work, a reduction in the amount of cholesterol in the plasma membrane and lipid rafts by statin treatments might cause the neurons to lose their shape due to the loss of connections between plasma membrane and the cytoskeleton. This connection is also related to the formation of blebs (Dai and Sheetz, 1999; Norman et al., 2010), which are spherical outgrowths of the plasma membrane that usually are formed during apoptosis (Yuan et al., 2003; Babiychuk et al., 2011). The formation of blebs occurs when the connections between the plasma membrane and the cytoskeleton are weakened (Doctor et al., 1997). The rougher and more jagged perimeters in the 30-day old neurons in statin-treated birds might also be related to the formation of blebs. Perhaps, the formation of blebs gave the neurons in the statin-treated birds an appearance of having rough and jagged perimeters, although we did not identify full blebs in our tissue.

One reason that statin treatment did not affect the morphology of older neurons may be because more membrane cholesterol is found in older neurons than in young neurons as suggested in an *in vitro* study (Nicholson and Ferreira, 2009). The effects of statins on membrane cholesterol may be more pronounced in young neurons than in older neurons, and may suggest that the amount of membrane cholesterol that statins affect is limited. Therefore, the larger amount of membrane cholesterol that older neurons have may conceal any effects that statins have on cholesterol reduction that would lead to morphological changes seen with 30-day old neurons. Moreover, if these morphological changes are signs of apoptosis, then it might
reflect the multiple mechanisms that older neurons have to prevent apoptosis (Review: Kole et al., 2013).

**Statins and neuronal size**

During song learning, songbirds compare their own song with the memory of the tutor’s song in order to produce a good song copy (Solis and Doupe, 2000). The number of neurons in HVC responding to playbacks of the bird’s own song is related to the quality of song learning such that birds that copy the tutor’s song well had more neurons activated in HVC (Bolhuis et al., 2012). Since new neurons can be activated easier by a stimulus than older neurons (Wang et al., 1999; Synder et al., 2001; Schmidt-Hieber et al., 2004), the relationship between song learning and the number of new neurons could also be positive. At this point, this idea has not yet been tested. If this relationship is true, it is possible that the new neurons in HVC store the memory of the bird’s own song that is necessary to produce a good song copy. Since there is a trend towards a significant lowered quality of song copying in statin treated birds, it is possible that the new neurons in HVC were affected by statin treatments.

Instead of finding an effect of statin treatment on the numbers of new neurons in HVC, we found that the effects were on the morphology of 30-day old new neurons and the sizes of the neurons that were relatively older than 30 days in HVC. It was reported that the soma sizes of LMAN neurons, RA neurons and HVC neurons increased during song development (Nixdorf-Bergweiler, 1998). HVC neurons reached their largest size when the birds were around 45 days old and then remained constant afterwards (Nixdorf-Bergweiler, 1998). If the older neurons were born during song learning, their soma sizes should remain constant. However, the older neurons in the statin-treated birds were smaller in area than those in control birds. One explanation for the decreased size could be that the older neurons were not able to grow
normally. Since cholesterol is an important neuronal component, the neurons might not be able to grow to reach the normal size due to the statins limiting available cholesterol. In addition, another reason for impairment of neuronal growth could be related to the effects of statins on glial cells, which supply cholesterol to mature neurons (Funfschilling et al., 2007). *In vitro* studies reported that statins decreased the survival of glial cells (Marz et al., 2007). In our work, it is possible that statin treatments would also affect the number of glial cells and the cellular components of the glial cells. In this case, the neurons would not have enough cholesterol to allow them to reach their normal size.
CHAPTER 7: LIPOPHILICITY OF STATINS AND MEMORY IN ADULT ZEBRA FINCHES (AIM 5)

Statins can be categorized based on whether they are lipophilic or hydrophilic. Lipophilic statins such as simvastatin can diffuse across the blood-brain barrier while hydrophilic ones such as pravastatin cross through an organic anion transporter, oatp1a4 (Fujii et al., 2015). Statins that can cross the blood-brain barrier can affect brain cholesterol (Vecka et al., 2004), which is an important neuronal component found in the plasma membrane and myelin sheath. Adult statin users have reported memory loss after taking statins, and symptoms were alleviated when they stopped taking them (Wagstaff et al., 2003; Evans and Golomb, 2009). However, the results are mixed and studies have also found no relationships between statin usage and cognitive impairments (Bettermann et al., 2012; Ott et al., 2015). Nevertheless, patients’ self-reports have prompted the FDA to issue warning labels related to the possible effects of statins on cognition. There have been many suggestions explaining how statins may affect cognition in humans (Lu et al., 2007; Wu et al., 2008). One idea is that lipophilic statins assert a greater effect on cognition than hydrophilic ones (Bettermann et al., 2012). This effect could be due to their ability to diffuse through the blood-brain barrier without transporters. However, another study found that the effects of statins on cognition were independent of the degree of lipophilicity of statins (Haag et al., 2009). Because of such the contradictory results, it is important to evaluate whether effects on cognition are independent of the lipophilicity of statins. The goal of this work was to determine whether statins with different degrees of lipophilicity affect memory and neuronal survival in adult birds. The results of this work may contribute to understanding potential cognitive and neural effects in humans.

Specific Methods
**Experimental design**

In this Aim, there were two experiments. In the first experiment, adult zebra finches were given either a clinical dosage of atorvastatin (Lipitor) or vehicle daily. This allowed us to determine whether atorvastatin affects the memory of juvenile birds (see Chapter 6) and the memory of adult birds differently. In the second experiment, a different set of adult zebra finches were given either a clinical dosage of hydrophilic pravastatin (Pravachol), simvastatin (Zocor), or vehicle and then underwent tests of auditory memory. This allowed us to determine whether the degree of lipophilicity of statins determines the statins effect on memory.

In both experiments, the birds received treatment every day for about 60 days. After 30 days of treatment, they received BrdU injections for 3 days (as described in General Methods). One day after the last day of statin treatment, electrophysiological recordings were done in NCM to measure the 20-hour memory of conspecific songs. On the following day, the birds were sacrificed and their brains were processed for immunohistochemistry to label and quantify 30-day old neurons in NCM (see Figure 22).

**Animals**

Adult birds (n = 34) were 82 - 480 days old (mean = 213 days, SEM = 20.6). Birds were housed in a group aviary, and remained in their cages throughout the duration of statin treatments and BrdU injections. After 60 days of statin treatment, each bird was then isolated in a sound attenuated box for 20-hour song memory recording.

**Statin administration**

Aim 4 consisted of two experiments. In the first experiment, adult male zebra finches were either given 40 mg/kg atorvastatin (Lipitor®, obtained from Pfizer) in 50 μl water (n = 7) or 50
μl water (n = 9) orally, daily for an average of 56 days. In the second experiment, another set of adult birds were either given 40 mg/kg of pravastatin (Pravachol®, obtained from Bristol-Myers Squibb Pharmaceuticals or Sigma Aldrich) in 50 μl water (n = 7), 40 mg/kg of simvastatin (Zocor®, obtained from Merck) in 50 μl water (n = 5), or 50 μl water (n = 6) daily for 60 days.

20-hour song memory

Procedures were the same as in Chapter 6.

Brain processing

We only used the left hemispheres because there was no a priori reason to think that the drugs might affect the two hemispheres differently. The birds were sacrificed one day after the last day of the statin treatment and brains were processed for immunohistochemistry (see General Methods for histology and immunohistochemistry).

Statistics

Mixed ANOVA (within subject variable = song familiarity and between subject variable = treatment) was used in parts 1 and 2 to determine whether there were any differences between the neuronal responses (Absolute Response Magnitude (ARM) and adaptation rate) in response to the familiar songs and to the novel songs and between the control birds and statin-treated birds. Two-tailed t-tests were used to compare RRS, FI and the number of new neurons in NCM between the control birds and the statin-treated birds in part 1. One-way ANOVA was used to compare RRS, FI and the number of new neurons in NCM between the control birds and the two statin-treated groups (simvastatin-treated birds and pravastatin-treated birds). Significance was set at p = 0.05 for all tests.
**Results**

**Part 1: Atorvastatin**

One of the statins associated with memory problems is atorvastatin (Lipitor). In the previous chapter (Chapter 6), we found that treating birds throughout the critical learning period did not affect the birds’ 20-hour memory of conspecific songs. In this follow-up work, our first goal was to determine whether treating adult birds with Lipitor would affect their 20-hour memory of conspecific songs. First, we compared the neuronal responses to familiar songs and novel songs between the control birds and the statin-treated birds. As expected, we found that there was a main effect of the neuronal responses for the familiar and novel stimuli when the control groups and statin-treated groups were combined, as measured by the ARM (Figure 23A) and adaptation rate (Figure 23B). When the control birds and the statin-treated birds were combined, the ARMs for novel songs were higher than the ARMs for familiar songs indicating less familiarity of the novel songs ($F (1, 16) = 5.938, p = 0.027$, Mixed ANOVA, Figure 23A). There was a trend towards a significant main effect in the statin-treated group ($n = 8, t (7) = -2.196, p = 0.064$, two-tailed paired t-test, Figure 23A) but not in the control group ($n = 10, t (9) = -1.110, p = 0.296$, two-tailed paired t-test, Figure 23A). The adaptation rates for the novel songs were also greater than the adaptation rate for the familiar songs ($F (1, 16) = 4.480, p = 0.050$, Mixed ANOVA, Figure 23B). However, there was no difference between the control group and the statin-treated group for both measurements (ARM: $F (1, 16) = 1.218, p = 0.286$, Mixed ANOVA, Figure 23A; Adaptation rate: $F (1, 16) = 0.123, p = 0.731$, Mixed ANOVA, Figure 23B). There was also no interaction between the treatment groups and song familiarity for both measurements (ARM: $F (1, 16) = 1.055, p = 0.320$, Mixed ANOVA; Adaptation rate: $F (1, 16) = 0.790, p = 0.387$, Mixed ANOVA).
When we used RRS and FI to measure the 20-hour memory of the familiar song, we also found that the neuronal memory was not different between the control birds and the statin-treated birds (mean +/- SEM, FI: Control (n = 10), 1.549 +/- 0.279; Statin-treated birds (n = 8), 1.631 +/- 0.177; t (16) = 0.234, p > 0.05, two-tailed t-test; RRS: Control (n = 10), 0.042 +/- 0.016; Statin-treated birds (n = 8), 0.041 +/- 0.025; t (16) = 0.011, p > 0.05, two-tailed t-test, p > 0.05, two-tailed t-test, Figures 24A, B).

Similar to the results from the quantification of new neurons in NCM in birds that were treated with statins throughout the critical period (Aim 4), atorvastatin treatment did not affect the survival of 30-day old new neurons in the adult birds (mean number per mm² +/- SEM, Control birds (n = 9), 4.073 +/- 0.941; Statin-treated birds (n = 7), 4.97 +/- 0.988; t (14) = 0.651, p = 0.526; two-tailed t-test, Figure 25).

Part 2: Simvastatin and Pravastatin

The goal of this second part of the experiment was to determine whether a lipophilic statin and hydrophilic statin affect memory differently. We first used the ARM to compare the neuronal memory in response to the familiar songs to the neuronal memory in response to the novel songs for each treatment group. We found that when memory was measured using ARM, there was a main effect of song type (familiar vs. novel) when the birds from all three groups (control, simvastatin-treated, pravastatin-treated) were combined (F (1, 28) = 10.561, p = 0.003, Mixed ANOVA, Figure 26). This effect was also found in control birds (n = 12, t (11) = -4.818, p = 0.0005, two-tailed paired t-test, Figure 26). However, it was not found in both statin-treated groups (Simvastatin-treated birds (n = 9, t (8) = -1.529, p = 0.165, two-tailed paired t-test; Pravastatin-treated birds (n = 10), t (9) = -0.242, p = 0.814, two-tailed t-test, Figure 26). ARMs were lower, indicating a memory, for familiar songs.
Interestingly, there was no difference between the control group and the statin-treated groups when neuronal memory was measured with ARM (F (2, 28) = 0.713, p = 0.499, Mixed ANOVA, Figure 26). These results indicate that the birds had a memory of the familiar songs regardless of the treatments the birds received. However, there was a trend towards a significant interaction between the treatment groups and song familiarity for ARM measurement (F (2, 28) = 2.968, p = 0.068, Mixed ANOVA), which may indicate that the differences in the neuronal responses for the familiar songs and the novel songs may depend on the statin treatments.

Adaptation rate is another way to measure neuronal memory. A slow adaptation rate, which is due to low neuronal responses, indicates a strong memory (Phan and Vicario, 2006; Tsoi et al., 2014). Overall, there was a main effect of song type (familiar vs. novel) when the birds from all three groups (control, simvastatin-treated, pravastatin-treated) were combined (Figure 27). For all three treatment groups, the adaptation rates responding to novel songs were faster than those responding to familiar songs (F (1, 28) = 4.40, p = 0.045; two-way mixed ANOVA, Figure 27). This indicates that all of the birds had stronger memory of the familiar songs than of the novel songs. There was also a main effect of treatment. The adaptation rates in response to the familiar songs and the novel songs were different among the three groups (F (2, 28) = 3.66, p = 0.039; mixed ANOVA). There was no interaction between the treatment groups and song familiarity (F (2, 28) = 0.142, p = 0.868, Mixed ANOVA).

When we further analyzed how the control, pravastatin, and simvastatin treatments differed from each other using Fisher’s Least Significant Difference (LSD) post-hoc tests, pravastatin-treated birds (n = 10) had a higher adaptation rate than the control birds (n = 12) during playback of familiar songs (mean +/- SEM, Control birds (n = 12), -0.25 +/- 0.02; Pravastatin-treated birds (n = 10), -0.36 +/- 0.06; p = 0.017), and novel songs (Control birds (n =
These results indicate that pravastatin-treated birds had a weaker memory of the familiar songs than the control birds.

We also used LSD post-hoc tests to compare the adaptation rates between the control birds and simvastatin-treated birds. We found that there was a trend toward simvastatin-treated birds having a higher adaptation rate in response to the playback of the familiar songs and the novel songs than the control birds (mean +/- SEM, Familiar songs: Control birds (n =12), -0.25 +/- 0.02; Simvastatin-treated birds (n = 9), -0.33 +/- 0.03; Novel songs: Control birds (n = 12), -0.27 +/- 0.02; Simvastatin-treated birds (n = 9), -0.37 +/- 0.04; p = 0.06 for both song types). These results suggest that simvastatin-treated birds had a trend toward a weaker memory of the familiar songs than the control birds.

The post-hoc test also revealed that there were no differences between the pravastatin-treated birds (n = 10) and the simvastatin-treated birds (n = 9) in adaptation rate responses to familiar songs (p = 0.63), suggesting that the more lipophilic simvastatin did not have a stronger effect on memory than the hydrophilic statin pravastatin. On the contrary, our data suggest that at least under our treatment conditions, hydrophilic statins may have a stronger effect on memory than the lipophilic statins since pravastatin-treated birds had significantly weaker memory than the control birds, and the differences between the control birds and the simvastatin-treated birds were not significant.

Twenty-hour memory can also be measured using RRS and FI, which calculate the relative difference of the ARM and the relative adaptation rates between the familiar songs and the novel songs, respectively. We found that the RRS and FI were not different among the three
treatment groups (FI: F (2, 49) = 1, p = 0.375, one-way ANOVA, Figure 28A; RRS: F (2, 49) = 2.02, p = 0.144, one-way ANOVA, Figure 28B).

Even though statin treatments impaired the birds’ 20-hour memories based on the adaptation rates in responses to the familiar songs and the novel songs, the numbers of new neurons in NCM were not affected (mean +/- SEM, Control (n = 5), 1.213 +/- 0.199; Pravastatin (n = 6), 1.308 +/- 0.35; Simvastatin (n = 5), 2.248 +/- 0.398; F (2, 13) = 1.24, p = 0.321, one-way ANOVA, Figure 29). These results suggest that memory effects may not be related to new neuron numbers since differences in memory impairment between lipophilic simvastatin and hydrophilic pravastatin did not correspond to group differences in numbers of new neurons in NCM.

**Discussion**

In this Aim, there were two separate studies. In the first study, adult zebra finches were treated with either atorvastatin (Lipitor) or vehicle for ~60 days to test whether statin treatments affected 20-hour song memory. We first measured memory by the Absolute Response Magnitude (ARM) and adaptation rates to playbacks of familiar and novel songs. We found from both ARM and adaptation rates that there was an overall main effect of the song familiarity such that the neuronal response and adaptation were higher to the playback of the novel songs than to the playback of the familiar songs when the birds in each group were combined.

Unexpectedly, control birds in the atorvastatin study did not show a memory for familiar songs. This might indicate that this set of birds may have required a longer exposure of the conspecific songs to show a familiarity effect. Further experimentation needs to be done to find out the reason for this result. Nevertheless, the data for control birds can still be used for comparison purposes. Based on our data, atorvastatin-treated birds had higher response
magnitude and adaptation rate than the control birds, although the differences were not significant. Our measurements of memory (ARM, adaptation rate, Relative Response Strength (RRS) and familiarity index (FI)) indicated that atorvastatin-treatments did not affect the adult birds’ neuronal memory, which was measured by ARM, adaptation rate, RRS and FI. Similarly, we found no effect of atorvastatin on 20-hour memory in birds treated with atorvastatin as juveniles throughout the critical learning period (Chapter 6).

In the second study, a separate set of adult zebra finches were given either pravastatin, simvastatin or vehicle for 60 days to test whether lipophilic statins and hydrophilic statins would affect 20-hour neuronal memory differently. In this study, we also measured 20-hour memory using ARM, adaptation rate, RRS and FI. Interestingly, we found different results depending on the testing method.

Using ARM and adaptation rate, we found an overall main effect of the song type (novel vs. familiar) when the birds in each group were combined, showing a memory for familiar songs. Unlike in the comparison between atorvastatin-treated and control birds, in this second study (comparing simvastatin-treated, pravastatin-treated and control birds), the controls did show the expected 20-hour memory for familiar songs. There was a significant difference within these control birds in the ARMs in response to familiar and novel songs. Based on the adaptation rates, pravastatin-treated birds had a weaker memory than the control birds, while there was no significant difference between simvastatin-treated birds and the control birds. However, we did not find any treatment differences in 20-hour memory among the control birds, simvastatin-treated birds and pravastatin-treated birds (measured using ARM, FI and RRS).

For both studies (parts 1 and 2), we did not find any effects of statins on the number of new neurons in NCM, which was found to be related to learning and memory (see Chapters 4
This result was not surprising for the atorvastatin study (part 1) since there were not any differences in 20-hour memory between the control birds and the atorvastatin-treated birds. However, it was unexpected when we found that simvastatin treatment and pravastatin treatment did not affect the number of new neurons in NCM even though pravastatin-treated birds had a weaker memory than the control birds.

**Statins and Memory**

Self-reports from adult patients suggest that simvastatin, atorvastatin, and pravastatin impair memory (Wagstaff et al., 2003; Suraweera et al., 2016). However, other studies report no relationships between statin usage and cognitive impairments (Bettermann et al., 2012; Jamolowicz et al., 2015; Ott et al., 2015). In our studies, we found that atorvastatin did not affect 20-hour neuronal memory in adult zebra finches while pravastatin (and a trend toward simvastatin) treatments impaired 20-hour neuronal memory in a separate set of adult zebra finches compared with their respective control groups.

A reduction of brain cholesterol by statins could potentially affect the number of new neurons, which are related to learning and memory (see Chapters 4 and 5). Therefore, memory impairment may be caused by the effects of statins on new neurons. In this work, only the number of new neurons was quantified. However, the numbers of new neurons in adult NCM were not affected by any of the statin treatments.

**Demyelination**

There are several other explanations linking statin treatment to impaired memory by means of reduced brain cholesterol. For instance, lowered available cholesterol may result in impaired axonal myelination. In the mammalian brain, the majority of brain cholesterol is found in the
myelin sheath (Dietschy, 2009), which is important for communication between neurons. Thus, reducing cholesterol may affect memory by interfering with neural signaling. It was found from postmortem multiple sclerosis brains that demyelination in the hippocampus reduced proteins that are important for anterograde and retrograde axonal transport, the number of synapses in the hippocampus, the number of glutamate receptors and glutamate transporters, and key molecules for learning and memory (Dutta et al., 2011). Perhaps, statins might directly affect these factors to impair cognition. Moreover, the structure of oligodendrocytes, which are important for remyelination, were also affected by statin treatments both in vitro and in vivo (Klopfleisch et al., 2008).

**Neurites**

Another explanation for our finding of memory impairment could be related to the effect of statins on axon and dendritic structure. However, findings from various studies are contradictory. While some statin treatments of neurons in vitro damaged neurites and inhibited growth (Pavlov et al., 1995; Schulz et al., 2004), other in vitro studies found that statin treatments promoted neurite growth (Sato-Suzuki and Murota, 1996; Pooler et al., 2006; Samuel et al., 2014). Currently, it is unknown whether statins promote or inhibit neurite growth in vivo.

**Synaptic vesicles**

Like the plasma membrane, the membrane of the synaptic vesicles that contain neurotransmitters are also composed of cholesterol. In fact, vesicles have a larger amount of cholesterol than other organelles (Pfrieger, 2003). An in vitro study reported that statin treatments decreased the amount of cholesterol in the plasma membrane and the number of synaptic vesicles released (Mailman et al., 2011). Statin treatments in our work might affect the formation of vesicles.
Such an effect was demonstrated *in vitro* when the formation of synaptic like microvesicles (SLMV) was decreased due to cholesterol reduction (Thiele et al., 2000). Moreover, cholesterol is also found to bind to the proteins in the vesicle membranes such as synaptophysin, and it is suggested that this association is important for the formation of SLMV (Thiele et al., 2000). Finally, statins might also affect the amount of neurotransmitter loaded into the vesicles by altering the stability of cholesterol-binding proton pump on the vesicles. This impairment has been demonstrated by an acute cholesterol reduction *in vitro* (Tarasenko et al., 2010) although another study showed that cholesterol reduction did not affect endocytic activity in SLMVs (Thiele et al., 2000).

**Effect of statins on NCM neurons**

In NCM, most neurons are GABAergic (Pinaud & Mello, 2007) and are activated by song playback (Jeong et al., 2011). Not all of the GABAergic neurons were activated, however, only those GABAergic neurons that have the α5 subunit (GABA5) were upregulated during auditory stimulation (Jeong et al., 2011). Therefore, the decreased auditory memory of familiar songs seen in the statin-treated birds may be caused by inhibition of the GABRA5 neurons. It was suggested that the GABRA5-expressing neurons may be involved in long-term memory but not involved in the initial adaptation of the stimuli (Jeong et al., 2011). Therefore, it is possible that the statin treatments affected the expression of this population of GABAergic neurons in NCM. It was shown *in vitro* that reduction of membrane cholesterol decreased neuronal responses to GABA, which was suggested to be involved with a specific binding of cholesterol to GABA<sub>A</sub> receptor (Sooksawate and Simmonds, 2001). Thus, statin treatments may lower the membrane cholesterol, which may alter the activity or the function of the GABAA5-expressing neurons through the alteration of the receptor.
*Lipophilicity and memory*

We first hypothesized that lipophilic stains would affect memory more than hydrophilic statins due to their ability to cross the blood-brain barrier easily. Surprisingly, however, we found the opposite. Compared to the control birds, pravastatin-treated birds had higher adaptation rates (worse neuronal memory). There was only a trending difference between the simvastatin-treated birds and the control birds, demonstrating that simvastatin treatments did not significantly affect the birds’ 20-hour memory. These results were similar to those of Stuart et al (2013), in which pravastatin-treated rats had impaired working and recognition memories compared to atorvastatin-treated rats. Our results support the idea that the degree of lipophilicity does not determine how statins affect cognition. The influx and efflux of pravastatin across the blood-brain barrier is assisted by oatp1a4, which is an organic anion transporter (Fujii et al., 2015). Thus, even though pravastatin does not diffuse through the blood-brain barrier, it still can lower brain cholesterol (Vecka et al., 2004).

Other work supports the idea that the effects of simvastatin and pravastatin on cognition do not appear to be associated with the lipophilicity properties of the drugs. In fact, these two statins affected expression of different genes and protein that are important for the regulation of memory and are associated with Alzheimer’s disease (Huber et al., 1993; Turner et al., 2003; Guan et al., 2011; Takashima, 2012; Liu et al., 2015). For example, only simvastatin increases the expression of GSK3β and CDK5, which regulate tau phosphorylation in neurons and only pravastatin increases the expression of amyloid precursor protein (APP) (Dong et al., 2009). Perhaps, the memory impairment observed in pravastatin-treated birds may be related to effects of statins on the expression of these genes.

*Isoprenoids*
The production of cholesterol in the liver involves multiple steps (see Figure 2). In this pathway, called the mevalonate pathway, the rate-determining step is the production of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) from acetylacetoyl-CoA with HMG-CoA reductase as the rate-limiting enzyme. It is this enzyme that statins inhibit to lower cholesterol production in the liver. Moreover, there are several intermediates such as mevalonate pyrophosphate, isopentenyl pyrophosphate, farnesyl pyrophosphate (FPP), and geranyl pyrophosphate (GGPP), produced in this pathway. FPP and GGPP, which are composed of lipids, are important for post-translational modification (isoprenylation) of small GTPases such as Ras, Rab and Rho, and are involved in cell signaling and cell survival (Hooff et al., 2008; 2010). Not only can statins reduce cholesterol production, they can also reduce the production of FPP and GGPP (Liao, 2002), which consequently reduces the amount of Rho and Rab (Segatto et al., 2014).

The reduction of the amount of these small GTPases may affect learning and memory. It was reported that the reduction of Rho GTPases impaired memory by affecting hippocampal neurotransmission and the regulation of the cytoskeletal dynamics (Diana et al., 2007). Unexpectedly, in the hippocampus, the reduction of RhoA, which is important for long-term potentiation, did not have a negative impact on cognition. In young rats, the reduction of RhoA increased the activation of CREB, which enhanced the formation of long term memory by the activation of Akt kinase (Segatto et al., 2014). This mechanism, however, does not explain our result of impaired memory. Interestingly, the inhibition of RhoA activity in the striatum was found related to impairments of working memory in old rats (Kang et al., 2013).

It is not known whether RhoA is involved in similar mechanisms in songbirds. However, it is possible that statin treatments could reduce RhoA, which is important for the stability of microtubules (Review: Wojnacki et al., 2014). Moreover, statins can also affect the development
of axons and dendrites as they are shown to be regulated by RhoA (Chen and Firestein, 2007; Kim et al., 2009).

Another small GTPase that is reduced by statins is Rab3 (via reduction in GGPP) which is important to the release of neurotransmitter from synaptic vesicles (Segatto et al., 2014). Therefore, neurotransmitters that are important to learning and memory could be reduced and communication between neurons would be affected.

Statin treatments in zebra finches could also affect the production of isoprenoids such as RhoA and Rab3 by blocking the mevalonate pathway. These isoprenoids are important for learning and memory through different mechanisms. To determine the role that isoprenoids have on the effects of statins in our birds, we could block the production of isoprenoids without interfering with the rest of the mevalonate pathway. If learning and memory is not affected, it is possible that other intermediates in the pathway are affected by statins.
CHAPTER 8: GENERAL DISCUSSION

Summary

Like human speech learning, songbirds require a model or a tutor to learn their songs (Doupe and Kuhl, 1999). During the sensory phase of song learning, songbirds listen to the tutor’s song and store the song memory in the avian secondary auditory cortex, called the caudomedial nidopallium (NCM, Bolhuis et al., 2000; Phan et al., 2006). This memory is then presumably used as a template for the bird to learn to sing his own song during the sensorimotor phase of song learning (Solis and Doupe, 2000; Mooney, 2009). NCM also stores memories of other conspecific songs in addition to the tutor’s song memory. Similar to previous findings (Phan and Vicario, 2010), we reported here that the left hemisphere NCM has a stronger memory of conspecific songs than the right hemisphere NCM.

NCM receives new neurons throughout the bird’s lifetime. Prior to the present work, it was known that auditory (Pytte et al., 2010) and social (Lipkind et al., 2002; Barnea et al., 2006) experience impacts numbers of new neurons in NCM. It was also established that the two hemispheres respond to conspecific songs differently (Phan and Vicario, 2010). However, it was not known whether there were hemispheric differences in the numbers of new neurons in NCM. We found that there are naturally more new neurons in the left hemisphere NCM than the right hemisphere NCM. Moreover, the lateralization of new neurons to the left hemisphere corresponded to the quality of song copying, which was measured by the similarity between the tutor’s song and the bird’s own song. This work, however, does not indicate that having more new neurons in the left hemisphere promotes better song copying or that song learning causes more new neurons to be incorporated into the left hemisphere NCM -- the causative nature of the correlation has not yet been tested.
The degree of lateralization of new neurons to the left hemisphere was also correlated with the strength of 20-hour neuronal memory of conspecific songs in the right hemisphere. In other words, birds that had fewer new neurons in the right NCM relative to the left NCM had a stronger neuronal memory in the right hemisphere. Although this is an interesting finding, the implications of the correlation between left-lateralization of new neurons and memory strength in the right hemisphere are not clear. If there is a functional relationship, it may be that the left hemisphere requires increased plasticity in the form of new neurons to process new information, whereas the right hemisphere requires increased stability of circuits (i.e. fewer new neurons) in order to maintain previously learned information. Although we do not yet understand the functional interplay between hemispheres, this is the first study to establish a relationship between lateralization in numbers of new neurons and learning and memory.

We then used these foundational findings of neurogenesis and memory to study the effects of statins in this system. Statins are commonly prescribed drugs used to lower the synthesis of cholesterol in the liver. However, statins can also cross the blood-brain barrier, and affect brain cholesterol (Locatelli et al., 2002; Kirsch et al., 2003; Vecka et al., 2004; Burns et al., 2006). Altering brain cholesterol could also affect brain function. In old rats, it was shown that the loss of cholesterol in the hippocampus impaired long-term depression (LTD), learning, and memory. These effects were reversed by infusing cholesterol into the brain (Martin et al., 2014).

Prior to this study, there were reports that statins cause memory impairments (Wagstaff et al., 2003; Sahebzamani et al., 2014); however, the claim is controversial (Bettermann et al., 2012; Ott et al., 2015). Given that neurogenesis is important to learning and memory (Snyder et al., 2005; Tashiro et al., 2007; Deng et al., 2009; Trouche et al., 2009; Aimone et al., 2011;
Vukovic et al., 2013; Akers et al., 2014), the effects of statins on new neurons in particular may be one of the explanations for memory loss suffered by some statins users. In addition to adult users, four types of statins are approved by the FDA for children with familial hypercholesterolemia (Stein, 2007).

In the first part of our tests of the effects of statins, we investigated how treating juvenile zebra finches with statins affected learning, memory, and new neurons in NCM -- tested in adulthood. We reported that even though juvenile birds treated with atorvastatin (Lipitor) did not have as strong a memory of the tutor’s song as the control birds, the success of song learning was not affected. Surprisingly, atorvastatin treatments did not affect the numbers of new neurons in NCM and or in HVC, a nucleus of the song motor pathway, but did affect the morphology of new neurons and soma size of older neurons in HVC. The neuronal memories for recently heard conspecific songs were also not affected by the treatments. Other regions were not examined.

In the second part of our tests of the effects of statins, we investigated whether statins with different degrees of lipophilicity differentially affected memory and numbers of new neurons in NCM of adult zebra finches. While lipophilic statins such as simvastatin can easily diffuse through the blood-brain barrier (Tsuji et al., 1993), the movement of hydrophilic statins such as pravastatin through the blood-brain barrier is mediated by oatp1a4 and oat3, which are organic anion transporters (Kikuchi et al., 2004). Overall, there are more transporters located in the abluminal side of the blood-brain barrier that is facing the brain than the luminal side that connects to the capillary (Fujii et al., 2015; Kikuchi et al., 2003). Both lipophilic and hydrophilic statins can also enter the brain by a monocarboxylic acid transporter, for which simvastatin acids have a higher affinity than pravastatin (Tsuji et al., 1993). From this information, we predicted that hydrophilic statins are less likely to enter the brain than lipophilic
ones. Moreover, if the likelihood of passage is equal between lipophilic statins and hydrophilic statins, the rate of entering the brain would be slower for hydrophilic statins due to the localization of the transporters in the blood-brain barrier.

Our examination of statin effects with respect to lipophilicity was divided into two experiments. The first experiment was designed to test the effect of atorvastatin (intermediate lipophilic) on numbers of new neurons in NCM and 20-hour neuronal memory of conspecific songs in adult birds. We found that neither variable was affected by the treatment. The second experiment was designed to test the effect of pravastatin (low lipophilicity) on numbers of new neurons in NCM and 20-hour neuronal memory of conspecific songs in adult birds. Birds that were treated with simvastatin (high lipophilicity) had trends toward memory impairment. Surprisingly, pravastatin impaired 20-hour neuronal memory more than simvastatin, but the difference was not significant. Neither pravastatin nor simvastatin had an effect on the number of new neurons in NCM. These results suggest that there are factors other than lipophilicity of statins and the quantity of new neurons that could affect memory.

**Comparisons between humans and songbird**

In humans, there is a left hemispheric language dominance for most (~90%) right-handed people (Shtyrov et al., 1998). In the language-associated cortical regions, there are cell size and anatomical hemispheric asymmetries, which might correspond to the functional dominance of the left hemisphere (Hutsler, 2002). In songbirds, both song production and song perception are lateralized in the brain, and the degree of lateralization is species-specific (George, 2010). In the zebra finch, control of song production is lateralized to the right hemisphere (Williams et al., 1992) and storage of song memories is lateralized to the left hemisphere (Phan and Vicario,
2010; Moorman et al., 2012). However, unlike in humans, much less is known about anatomical asymmetry in the song system of songbirds’ brain.

In the current work, we found there are hemispheric differences in the numbers of new neurons in NCM, which is functionally analogous to the Wernicke’s area in the human brain. This is the first time that lateralization of new neurons has been reported in any species or brain region. Furthermore, it is possible that numbers of new neurons in the song system (HVC and Area X) are also lateralized due to the likely connections between the auditory system and the song system. This is currently being investigated. However, there are differences between NCM and Wernicke’s area. In particular, while there are new neurons incorporated into NCM throughout the bird’s life time, adult neurogenesis does not occur in the Wernicke’s area.

Due to the similarities between speech learning and song learning (Doupe and Kuhl, 1999; Gobes and Bolhuis, 2007) and homologies between the song system and mammalian circuits (Brainard and Doupe, 2013), our findings are relevant to humans and allow us to understand how statins could affect neural development in children.

**Lateralization of new neurons and memory**

Rodent studies have illustrated multiple relationships between new neurons and various aspects of learning and memory (Gould et al., 1999; Döbrössy et al., 2003; Dalla et al., 2007; Tashiro et al., 2007; Shors 2008; Trouche et al., 2009; Aimone et al., 2011; Curlik and Shors, 2011; Curlik et al., 2013; Akers et al., 2014). In rodents, the strength of memory is correlated with the number of new neurons in the hippocampus (Sisti et al., 2007), although the hemisphere has not been differentiated in the rodent studies. In songbirds, the relationship between new neurons and strength of neuronal memory of the song was not known. I hypothesized that the relationship in birds would be similar to the one in rodents. However, surprisingly, neuronal memory strength
was only correlated with the lateralization of new neurons in NCM (the relative difference between the two hemispheres) and not the absolute number of new neurons. This novel finding demonstrates that there is not a one-to-one relationship between memory strength and the number of new neurons – either within a single hemisphere or across both hemispheres.

However, we cannot conclude that the strength of neuronal memory of the conspecific songs corresponds directly to memory on a behavioral level. In our work, we did not perform any tasks to determine the relationship between electrophysiology and behavior. The birds that have better neuronal memory of the conspecific songs in NCM might not actually perform better in behavioral memory tasks of other birds’ songs. In addition, perhaps memory for other’s songs may involve the bird’s memory of its own song, which is stored in HVC (Bolhuis et al., 2012).

**The relevance of our work in understanding the potential effects of statins on humans**

Despite uncertainties about the effects of statins on cognition in adults (e.g., Wagstaff et al., 2003), statins were also approved for children who are genetically predisposed to high cholesterol (Stein, 2007). However, studies of the effects of statins in children were limited to measuring their school performances and sexual development (de Jongh et al., 2002; Wiegman et al., 2004) and did not provide a direct answer to whether statins may affect children’s neural and cognitive development. To my knowledge, there has been no animal research conducted on the effects of statins on juvenile models.

**Effects of statins in healthy brains**

There is a vast body of literature in animal models investigating the neuroprotective properties of statins on diseased or injured brains. The general conclusion is that statins may mitigate secondary effects of brain injury by reducing inflammation in the brain, decreasing cell death,
and increasing neurogenesis (Paintlia et al., 2005; Lu et al., 2007; Wu et al., 2008; Li et al., 2009; Krisanova et al., 2012; Kurata et al., 2012; Yuksel et al., 2013; Saito et al., 2014). However, animal research studying the effects of statins on healthy brains is limited to a few reports (Baytan et al., 2008; Stuart et al., 2013; Maggo and Ashton, 2014; Robin et al., 2013; Schilling et al., 2014). Due to the differences in treatment time, dosages and measurement methods among these studies, including ours, it is difficult to draw a general conclusion about how statins affect healthy brains.

**Lipophilicity does not predict effects**

Compared to control birds, birds that were treated with (hydrophilic) pravastatin, but not (lipophilic) simvastatin, had impaired neuronal memory of conspecific songs (Chapter 7). Our result, which is comparable to that of Stuart et al. (2013, in mice), illustrated that the degree of lipophilicity of statins does not determine its effects. This result is even more interesting because the dosage of 40 mg/kg of pravastatin is not as strong as the dosage of 40 mg/kg of simvastatin. Based on the amount of low density lipoprotein (LDL) reduced, 40 mg/kg of pravastatin is equivalent to 20 mg/kg of simvastatin (Smith et al., 2009). Therefore, if we only looked at both pravastatin’s and simvastatin’s effects on brain cholesterol level, the simvastatin-treated birds might be more affected than the pravastatin-treated birds. Our results further demonstrated that pravastatin might affect memory in other ways.

**Comparison between the current work and in vitro studies**

Many studies on healthy brain tissue have been conducted in vitro (Pavlov et al., 1995; Michikawa and Yanagisawa, 1999; Tanaka et al., 2000; Marz et al., 2007; Xiang and Reeves, 2009). Treating cultured neurons and glial cells with lovastatin and mevastatin resulted in
damaged plasma membranes due to suppression of cholesterol synthesis (Pavlov et al., 1995; Michikawa and Yanagisawa, 1999). Others have shown that morphological effects of atorvastatin and simvastatin on cultured glial cells were due to GGPP reduction, but not cholesterol reduction (Marz et al., 2007). Thus, GGPP reduction (in addition to, or independent of reduction in brain cholesterol) may underlie our findings of cell membrane damage as well.

It has been reported that *in vitro* statin treatments directly affected the numbers of axonal and dendritic branches (Fan et al., 2002), spine density and morphology (Hering et al., 2003), dendritic arborization (Kim et al., 2009) as well as other measures of damage to processes (Pavlov et al., 1995; Michikawa and Yanagisawa, 1999). In our work, we traced the contours of the neurons to determine the effects of statins on neuronal morphology. To my knowledge, this is the first time in which the effects of statins on cell morphology *in vivo* has been studied. In addition, there are no reports of any measurements quantifying morphological changes in the neuronal soma following exposure to statins *in vitro*. Interestingly, our findings of soma effects were similar to the observations described in *in vitro* studies based on detailed descriptions and photographs of the effects of statins on the morphology of the neural cells, although these effects were not quantified (Pavlov et al., 1995; Michikawa and Yanagisawa, 1999; Marz et al., 2007). By quantifying tracings of the contour of the neuronal cell bodies, our findings provide additional information about how statin treatments might affect neurons *in vivo* and potentially in humans.

In addition to affecting neuronal membranes, statin treatment *in vivo* may also affect axons and dendrites. Since the majority of brain cholesterol is found in the myelin sheath (Dietschy, 2009), the effects of statins on axons might be more pronounced than the effects on the cell membrane. Therefore, statins might also have affected the structures of axons and
dendrites in our experiments. In our tissue, we could not observe whether statin treatments affected neuronal processes for two reasons. First, the processes on most neurons were cut off because the tissue was sectioned into very thin (6 µm) sections. Second, the neuronal processes were not labeled with our methods. We only labeled the cell nucleus and soma cytoplasm.

**Future Directions**

In our work, we established the relationship between the lateralization of new neurons in NCM and learning and memory. Since this is only a correlational study, it is important to determine whether the success of song learning is determined by the lateralization of new neurons in NCM, or vice versa, or whether there is no functional relationship between the two variables. One possible way to approach this issue is to utilize *in vivo* optical imaging as Couillard-Despres et al. (2008) described. This technique allowed the researchers to track the growth and development of implanted progenitor cells. Using this technique, we could investigate whether implanting more progenitor cells in one hemisphere than in the other hemisphere would affect the success of song learning and the strength of neuronal memory. We could also use this technique to monitor the effects of statins on the development of young neurons as well as their migration and integration patterns.

It is important to investigate the balance between neurogenesis and apoptosis in NCM under normal conditions. The relationship between these two processes has only been studied in the song system in HVC (Scharff et al., 2000; Thompson and Brenowitz, 2009; Larson et al., 2014). Apoptosis has not yet been studied in NCM. It would be interesting to see whether apoptosis is also lateralized in NCM. In this current work, we found that the birds with more new neurons in the left NCM than the right NCM had better song copies. If the relationship between neurogenesis and apoptosis in NCM is the same as the one in HVC, maybe the birds
that can copy the tutor’s song well also had more apoptotic neurons, or a higher rate of turnover in the left NCM than the right NCM.

We found that statins affected the morphology of new neurons and the size of older neurons in HVC by tracing the soma of the neurons. *In vitro* studies showed that neurites were fragmented and lost when the neurons were treated with statins (Pavlov et al., 1995; Michikawa and Yanagisawa, 1999; Schulz et al., 2004). Others reported that statins inhibited the dendritic growth in neurons of adult rats both *in vitro* and *in vivo* (Kim et al., 2009). In order to determine whether statin treatments also affected dendrites and axons *in vivo* in songbirds, brain tissue would need to be cut into thicker sections and processed using a Golgi stain, which can also allow us to see the morphological effects that statin treatments might have on neurites as well as the synaptic connections between neurons.

In addition to neurons, it is also possible that statins affect glia cells. Marz et al. (2007) reported that cultured astrocytes that were given simvastatin and atorvastatin were morphologically different from the control cells. The effects of statins on the growth and development of oligodendrocytes and astrocytes, which are involved in the production of brain cholesterol, might provide an explanation for statins’ effects on the morphological changes of new neurons in our work.

Another type of glial cell that would be interesting to quantify is microglia. The morphologically altered neurons from statin-treated birds might be viewed as foreign and thus increase the number of activated microglia. Moreover, an *in vitro* study reported that simvastatin treatments increased the release of inflammatory mediators such as interleukin-1β (IL1β), tumour necrosis factor - α (TNFα) and brain-derived neurotrophic factor (BDNF) in activated microglia that were exposed to bacterial lipopolysaccharide (LPS) through cholesterol dependent
mechanisms (Churchward and Todd, 2014). By measuring the amount of these mediators released from activated microglia in our tissue, it might be possible to determine how the morphologically altered neurons affect microglia compared to the effects with LPS. However, at the same time, statins might activate anti-inflammatory responses from microglia due to their neuroprotective properties as seen with injured or diseased brains (Paintlia et al., 2005; Li et al., 2009; Saito et al., 2014; Kurata et al., 2012). By quantifying microglial activation, we might be able to determine whether statins are neuroprotective or neurotoxic to a healthy brain.

Furthermore, the new neurons which have morphological abnormalities after statin exposure might be more susceptible to apoptosis. Therefore, it is important to assess whether statins would increase apoptosis in vivo as they did in vitro (Pavlov et al., 1995; Marz et al., 2007). During an early stage of apoptosis, phosphatidylserine, which is a phospholipid, moves from the inner layer of the plasma membrane to the outer layer (Review: Schlegel and Williamson, 2001). Phosphatidylserine is important for the localization of cholesterol in the inner layer of the plasma membrane such that without it, cholesterol would be retained in the outer layer (Maekawa and Fairn, 2015). It is uncertain whether cholesterol could affect the distribution of phosphatidylserine the same way it did on cholesterol. However, since lipophilic statins reduced cholesterol in the inner layer of the plasma membrane (Kirsch et al., 2003; Burns et al., 2006), it would be interesting to see if lipophilic statins would speed up the process of apoptosis by affecting the distribution of phosphatidylserine indirectly. If there was an increased number of apoptotic cells in statin-treated birds, the number of activated microglia might also increase to phagocytize apoptotic cells.
FIGURES AND TABLES

A

B

Figure 1. Sagittal view of the songbird brain. (A) White arrows show the anterior forebrain pathway that is involved in song learning and maintenance. Black arrows show the motor pathway that is involved in song production (from Bauer et al., 2008). (B) Arrows show the ascending auditory pathway. Regions in yellow belong to the primary and secondary auditory cortices (from Bolhuis et al., 2012).
Figure 2. Cholesterol production pathway in the liver. Inhibition of HMG-CoA reductase by statins will affect the downstream production of cholesterol and isoprenoid proteins such as Rho and Rac. Figure adopted from Menge et al., 2005.
Figure 3. Hemispheric asymmetry in new neuron incorporation in NCM. (A) The left hemispheric NCM had significantly more new neurons than the right hemispheric NCM. Each black dot represents the density of new neurons in the left NCM. Each gray dot represents the density of new neurons in the right NCM. The black bars indicate the means. (B) 71% of the birds (22/31) had more new neurons in the left hemisphere. In the Neuron Asymmetry Index, positive numbers indicate there were more new neurons in the left hemisphere, negative numbers indicate there were more new neurons in the right hemisphere, and zero means there is no difference in the numbers of new neurons between the left and right hemispheres.
Figure 4. The number of new neurons in the left hemisphere was correlated with the number of new neurons in the right hemisphere. Each point represents an individual bird. The dashed line indicates the numbers of new neurons if both hemispheres were equal. Birds above the dashed line had more new neurons in the left hemisphere whereas birds below the dashed line had more new neurons in the right hemisphere. The orange line shows the best-fit regression which indicates higher new neuron density in the left hemisphere across the population. The orange arrow points to the y-intercept of 283.6.
Figure 5. Packing Density in NCM. Density of neurons of all ages (BrdU+/NeuN+; BrdU-/NeuN+) was not different between the left and right hemispheres. Each dot is one bird. The bar is the mean of each group.
Figure 6. There was no correlation between densities of new neurons and the quality of song copying when the left and right hemisphere was considered separately or combined. However, the relative difference in new neuron density is correlated with the quality of song copying. (A) There was a trend towards a significant correlation between new neurons in the left hemisphere and Similarity Index. (B) There was no correlation between the number of new neurons in the right hemisphere and the Similarity Index. (C) There was no correlation between the number of new neurons in both the left and right hemispheres and the Similarity Index. (D) The “Neuron Asymmetry Index” (NAI) was positively correlated with the Similarity Index in the complete set of birds. A positive number in NAI indicates that the left hemisphere has relatively more new neurons than the right hemisphere. A negative number indicates more new neurons in the right hemisphere. The lines are best-fit regression lines.
Figure 7. The correlations between new neurons and the quality of song copying when values for siblings were combined show the same pattern as when the birds were considered individually. There were 14 birds with at least one sibling in the entire set of birds. Since there is a genetic component in the number of new neurons, values for song copying and numbers of new neurons were averaged among brothers (Total n = 14). (A) There was a trend towards a significant correlation between new neurons in the left hemisphere and the Similarity Index. (B) There was a trend towards a significant correlation between new neurons in the right hemisphere and the Similarity Index. (C) There was no correlation between the number of new neurons in both left and right hemispheres and the Similarity Index. (D) NAI and Similarity Index were correlated with each other. (E) For birds in figure D, 11 birds that were trained with a live tutor. NAI and Similarity Index were also significantly correlated in this subset of live-tutored birds. The lines are the best-fit regression lines.
Figure 8. Both left and right hemispheric NCM had a memory of the conspecific song but the left hemispheric NCM had a significantly higher relative response rate than the right NCM. The variability of the RRS in the right hemisphere was greater than the variability of the RRS in the left hemisphere. The low RRS in the right hemisphere was driven by the negative RRS. This difference in RRS between the left and right hemispheric NCM was seen when (A) the birds were considered individually and (B) when the brothers were combined. Each black dot represents the RRS in the left NCM for one bird. Each gray dot represents the RRS in the right NCM for one bird. The means of each group are indicated by the black bar.
Figure 9. There was no relationship between the number of new neurons and the strength of 20-hour memory. The left RRS was not significantly correlated with the number of new neurons in the left NCM (A) when birds were considered individually and (B) when the brothers were combined. The right RRS was not significantly correlated with the number of new neurons in the right NCM (C) when birds were considered individually and (D) when the brothers were combined. The lines are best-fit regression lines.
Figure 10. The relationships between the degrees of lateralization of number of new neurons in NCM and memory strength were different between the left and right hemispheric NCM. RRS in the left hemisphere was not correlated with the lateralization of new neurons (A) when the birds were considered individually and (B) when the brothers were combined. (C) When the birds were considered individually, RRS in the right hemisphere was not correlated with the lateralization of new neurons. (D) When the brothers were combined, RRS in the right hemisphere was correlated with the lateralization of new neurons. The lines are best-fit regression lines.
Figure 11. Timeline for Aim 3 in which we investigated the effects of statin treatments in juvenile zebra finches on song learning, memory, and neurogenesis.
Figure 12. Across 11 song features, statin-treated birds’ songs were not different from the control birds’ songs. 10 song motifs were randomly selected for each bird. Each dot is one bird. Blue dots = Control birds; Green dots = Atorvastatin-treated birds. The black bars indicate the means of each group of birds in each condition.
Figure 13. Statin-treated birds had lower quality of song copy than control birds based on Similarity Index. Each bar represents an individual bird and was arranged from the lowest score to the highest score in each group. A high Similarity Index indicates a high success of song copying. The dashed lines indicate the average similarity score for each treatment group. Error bars = SEM.
Figure 14. The birds’ own songs and their tutor’s song were not different based on the comparisons across the 5 acoustic parameters that comprise the Similarity Score. For each bird, 10 song motifs were randomly selected and averaged. The averages for each group (control vs. statin-treated) were then compared to Samba. Each dot is one bird. Blue dots = Control birds; Green dots = Atorvastatin-treated birds. The black bars indicate the means of each group of birds in each condition.
Figure 15. The control birds had stronger neuronal memory of the tutor’s song than the statin-treated birds. Blue dots = Control birds; Green dots = Atorvastatin-treated birds. The black bars indicate the means of each group of birds in each condition. Dashed line indicates that the memory for the familiar song equals to the memory for the novel song.

Figure 16. Neuronal memory of the tutor’s song (measured with FI) was more related to the quality of song copying (measured with SI) in control birds than in statin-treated birds. Blue dots = Control birds; Green dots = Atorvastatin-treated birds. The dashed lines are the best-fitted regression line.
Figure 17. **Statin treatments did not affect 20-hour auditory memory of conspecific songs.** (A) Familiarity index (FI), which compares the neuronal activity in NCM in response to novel songs with the response to familiar songs, was not different between control birds and statin-treated birds. (B) The relative response strength (RRS), which measures the difference of neuronal firing activity in response to familiar song and novel songs, was not different between control birds and statin-treated birds. Blue dots = Control birds; Green dots = Atorvastatin-treated birds. The black bars indicate the means of each group of birds in each condition.
Figure 18. Atorvastatin did not affect the numbers of new neurons in NCM and HVC. (A) Numbers of 30-day old neurons in NCM. (B) Numbers of 30-day old neurons in HVC. (C) Numbers of 1-3-weeks old neurons in HVC. Blue dots = Control birds; Green dots = Atorvastatin-treated birds. The black bars indicate the means of each group of birds in each condition.
Figure 19. (A) There was a trend towards significance in the correlation between the numbers of 1-3 weeks old new neurons and the SI values in statin-treated birds but not in control birds. (B) Numbers of 30-day old new neurons were not correlated with SI in both groups of birds. Blue dots = Control birds; Green dots = Atorvastatin-treated birds. The dashed lines represent the best-fit regression lines.
Figure 20. New 30-day old neurons in statin-treated birds were morphologically different from those in control birds. (A) and (B) Neurons in statin-treated birds were flatter than those in control birds as indicated by low aspect ratio and roundness values. (C) Neurons in statin-treated birds were less compact than those in control birds. (D) Contours of 30-day old neurons in statin-treated birds were more convoluted than those in control birds as measured by shape factor. (E) Neurons in statin-treated birds had a rougher and more jagged membrane than those in control birds as indicated by low form factor values. (F) Soma sizes of neurons in statin-treated birds were the same as those in control birds. Blue dots = Control birds; Green dots = Atorvastatin-treated birds. The black bars indicate the means of each group of birds in each condition.
Figure 21. Older neurons in statin-treated birds had smaller areas and shorter diameters than those in control birds. (A) Soma area (B) maximum diameter across the center of the neuron (C) minimum diameter across the center of the neuron. Blue dots = Control birds; Green dots = Atorvastatin-treated birds. The black bars indicate the means of each group of birds in each condition.
Figure 22. Timeline for Aims 4 and 5 in which we investigated the effects of statin treatment in adulthood on memory and neurogenesis. Day 0 indicates the beginning of the experiment.
Figure 23. There was a main effect of song type (familiar songs vs. novel songs) in the statin-treated group. This effect was also found when the control birds and statin-treated birds were combined. Atorvastatin treatment did not affect 20-hour song memory. (A) 20-hour memory was measured by absolute response magnitude. (B) 20-hour memory was measured by the adaptation rates. Blue dots = Control birds; Red dots = Atorvastatin-treated birds. The black bars indicate the means of each group of birds in each condition.
Atorvastatin treatments did not affect 20-hour auditory memory of conspecific songs. (A) Familiarity index (FI), which compares the neuronal activity in NCM in response to novel songs and familiar songs, was not different between control birds and atorvastatin-treated birds. The dashed line indicates that the memory for the familiar song equals the memory for the novel song. (B) The relative response strength, which measures the difference of neuronal firing activity in response to familiar song and novels songs, was not different between control birds and atorvastatin-treated birds. Blue dots = Control birds; Red dots = Atorvastatin-treated birds. The black bars indicate the means of each group of birds in each condition.
Figure 25. Atorvastatin treatments did not affect the numbers of 30-day old new neurons in NCM. Blue dots = Control birds; Red dots = Atorvastatin-treated birds. The black bars indicate the means of each group of birds in each condition.
Figure 26. There was an overall main effect of song type (familiar songs vs. novel songs). There were no differences in 20-hour song memory between treatment groups. 20-hour memory was measured by the absolute response magnitude (ARM). When all of the birds were combined, the ARM in response to playbacks of familiar songs was significantly lower than the ARM responding to playbacks of novel songs. Each dot is one bird. Blue dots = Control birds; Grey dots = Simvastatin-treated birds; Purple dots = Pravastatin-treated birds; The black bars indicate the means of each group of birds in each condition.
Figure 27. Based on adaptation rates, there was a main effect of song type (familiar songs vs. novel songs) on 20-hour memory when all of the birds were combined. The adaptation rates in response to the novel songs were lower than those responding to the familiar songs for all three treatment groups. Adaptation rates were significantly different between the control group and the pravastatin-treated group. Pravastatin-treated birds had a higher rate (more negative number) than control birds for both song types. Each dot is one bird. Blue dots = Control birds; Grey dots = Simvastatin-treated birds; Purple dots = Pravastatin-treated birds; The black bars indicate the means of each group of birds in each condition.
Figure 28. Based on (A) Familiarity Index (FI) and (B) Relative Response Strength (RRS), statin treatments did not affect 20-hour memory of conspecific songs. Each dot is one bird. Blue dots = Control birds; Grey dots = Simvastatin-treated birds; Purple dots = Pravastatin-treated birds; The black bars indicated the means of each group of birds in each condition.
Figure 29. Statin treatments did not affect the numbers of new neurons in NCM. Each dot is one bird. Blue dots = Control birds; Grey dots = Simvastatin-treated birds; Purple dots = Pravastatin-treated birds. The black bars indicate the means of each group of birds in each condition.
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<th>Statin</th>
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Table 1. Statins are listed in order of degree of lipophilicity with mevastatin as the most lipophilic.
Table 2. There were no differences between the acoustic features of the tutor’s song (SAMBA) and the features of the experimental birds (Control birds and Atorvastatin-treated birds). For each bird, 10 song motifs were randomly selected and averaged. The averages for each group (control vs. statin-treated) were then compared to SAMBA. Shown are means +/- SEM.

<table>
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<tr>
<th></th>
<th>Pitch</th>
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<th>Amplitude Modulation</th>
<th>Wiener Entropy</th>
<th>Goodness of Pitch</th>
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<td>1.691 +/- 0.065</td>
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<td>3.156 +/- 0.121</td>
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<td>3.146 +/- 0.130</td>
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<td>0.95</td>
<td>0.84</td>
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REFERENCES


