2010

Effects of Estrogen on Recovery of Spatial Function After Cerebellar Lesion

Gerald William Stinson Jr.

Effects of Estrogen on Recovery of Spatial Function After Cerebellar Lesion

By

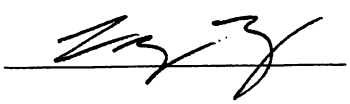
Gerald William Stinson Jr.

A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College

Oxford

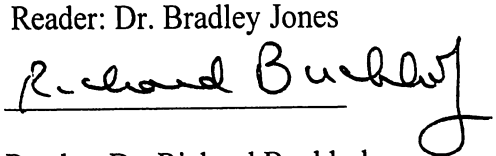
May 2010

Approved by



Advisor: Dr. Elaine Day

Reader: Dr. Bradley Jones



Reader: Dr. Richard Buchholz

Acknowledgements

This process has been a long one with both up and downs. I have many people to thank for my success in this endeavor who have all contributed to my growth, as a person and as a researcher.

First I would like to thank Dr. Lainy Day, my thesis advisor for allowing me to work in your lab. You have not only been a thesis advisor but a great mentor and life coach. You never gave up on me and always pushed me to do my best. Working in your lab has been an experience that I will never forget. The people in the lab, Madeline Coltharp, Josh Hamer, Amber Hancock, Nathan Parrott, Megumi Mathis, Buck Tramel, and Aaron Poole, were great and I thank each of you. Your help made this a successful project. Thank you to IMAGE MAMP (Mississippi Alliance for Minority Participation) staff, my mentors and tutors. The IMAGE program has really helped to make the University of Mississippi my home by forming a large support system for STEM majors. I would also like to thank the Honors College and Mississippi Functional Genomics Network for financially supporting me during my thesis process. I want to recognize my parents, Gerald and Daisy Stinson who have supported me both emotionally and financially. Most of all I would like to thank my Lord and Savior, Jesus Christ because without him none of this would have been possible.

Abstract

The traditional view of cerebellar processing has been that it plays a role in motor planning and function, now the cerebellum is also believed to be involved in spatial learning and cognition. Like the cerebellum's involvement in cognition the role of estrogen in cerebellar functioning has only recently been investigated. The cerebellum normally has low levels of estrogen but aromatase activity is upregulated after brain injury, increasing estrogen levels. This upregulation after injury suggests that estrogen could be involved in neuroprotection. This study uses male zebra finches to investigate the role of the cerebellum in spatial function and the possible role of estrogen in recovery of function after cerebellar lesion. To test the hypotheses that estrogen aids in recovery of spatial abilities after cerebellar lesion, we developed a maze for small birds to test spatial abilities. To examine recovery of function, I made bilateral puncture lesions to the cerebellum or performed a sham lesion (controls). I compared sham birds, to birds with bilateral lesions to the cerebellum, either given a control vehicle or fed vehicle + letrozole to block estrogen synthesis. Our findings suggest that the cerebellum is involved in spatial function and that estrogen improves the outcome of behavioral recovery after cerebellar lesions.

TABLE OF CONTENTS

Introduction

While the traditional view of the cerebellum has been centered on its involvement in motor function, recently there is evidence for the involvement of the cerebellum in cognition (Ebner & Pasalar, 2008; Glickstein & Doron, 2008). For example, there are correlations between abnormalities of the cerebellum and neurological diseases that cause cognitive impairments in humans (Steinlin, 2007). Further evidence comes from rodent studies, where lesioning of the cerebellum has resulted in impairments in cognitive abilities (Lalonde & Strazielle, 2003; Petrosini, 2007). Although the size and shape of the cerebellum differs greatly among species, the circuitry and connections of the cerebellum are conserved. In all vertebrates, the cerebellum has three cell layers; the molecular layer, granule layer and the Purkinje layer. The granule layer is the input layer and the Purkinje layer is the output layer. The regular and intricate input and outputs make it an ideal computer of timing and sequence of behaviors. Because its morphology is conserved, studies of the cerebellum in one species can be applied to other species (Spence *et al.*, 2009).

Although there are many causes of cerebellar dysfunction both natural and accidental, one known cause of age related declines in cerebellar function is a reduction in steroid hormones (Foy *et al.*, 2000). Steroid hormones are known to play a role in normal brain physiological processes (Moore & Evans, 1999), and may play a role in repair and recovery of function after damage to the cerebellum (Spence *et al.*, 2009) and other areas of the brain (Peterson *et al.*, 2001; Peterson *et al.*, 2004; Kelly *et al.*, 2008; Soderstrom *et al.*, 2009). The role of estrogen in cerebellar function has been far less studied than the role of estrogen in other brain areas. This is due, in part, to the fact that

under normal conditions there is little estrogen in the cerebellum, and there is no estrogen receptor alpha in the cerebellum (Ball *et al.*, 1999). Recently, however, a new estrogen receptor, estrogen receptor beta, was discovered, and it has been found that estrogen receptor beta is present at low levels in the cerebellum (Ball *et al.*, 1999). Estrogen's possible role in neuroplasticity in the cerebellum is particularly interesting because although there is normally little estrogen in the cerebellum, after traumatic brain injury aromatase is upregulated. Aromatase is the enzyme that converts androgens to estrogen. This raises the estrogen levels in the cerebellum around the lesion cite suggesting that estrogen may play a role in neuroprotection by preventing further cell death around the lesion (Spence *et al.*, 2009) and in neural repair after lesions (Foy *et al.*, 2000). This neuroplasticity may then serve to improve behavioral deficits caused by the lesions. However, the role of estrogen in recovery of function after damage to the cerebellum is even less well studied than the role of estrogen in repair and regeneration at the neural level. Thus, in my thesis research, I examined whether lesions of the cerebellum impair spatial ability and whether estrogen could improve recovery of spatial function after lesions in zebra finches. While a role of the cerebellum in spatial working memory had previously been shown in zebra finches (Spence *et al.*, 2009), no study has tested whether lesions of the zebra finch cerebellum interfere with spatial reference learning and whether estrogen could improve learning and memory outcomes after such lesions. We used zebra finches as a model for our studies as their brains are more plastic than mammals as will be further detailed below (Spence *et al.*, 2009). However, because cerebellar structure and many functions are conserved, our results should be applicable to other vertebrate models as well (Spence *et al.*, 2009). I review evidence below that

demonstrates that the cerebellum is involved in cognition across taxa and that estrogen plays a role in cerebellar function in a variety of taxa.

Cerebellar Function

Humans. In humans cerebellar abnormalities are associated with a variety of impairments. In children with cerebellar abnormalities there is a reduction in cognitive processing and cognitive processing speed (Steinlin, 2007). These children have lower IQ scores and have problems with visuospatial task (Steinlin, 2007). Patients with fragile X syndrome and Down's syndrome have smaller cerebellums than controls. People who suffer from William's syndrome, a neurodevelopmental disorder that causes mental retardation and visual spatial disabilities, have larger cerebellums when compared to controls (Steinlin, 2007). It is believed that cerebellar defects at birth lead to dyslexia (Steinlin, 2007). Dyslexic patients have difficulties reading and spelling and adults with dyslexia have implicit learning complications. However, this disorder is not related to intelligence level. Researchers believe that dyslexia could be related to defects of the cerebellum because the cerebellum differs more in dyslexia patients than in controls (Steinlin, 2007). These neurological disorders support the notion that cerebellar abnormalities interfere with cognitive function.

Damage to the cerebellum in humans also causes specific deficits in spatial learning. Patients with damage to the cerebellum have impairments in three spatial tests: the Raven standard progressive matrices, Ray complex figure, and block design (Lalonde & Strazielle, 2003). The Raven standard progressive matrices test is a multiple choice test using graphic designs to test the spatial and cognitive abilities of the test taker

(Prabhakaran *et al.*, 1997). The Ray complex figure task is a task where the subject has to draw a figure twice. The first time the figure is drawn by tracing, and the second figure is drawn from memory to test the visiographic memory of the patient (Liberman *et al.*, 1994; Hubley & Jassal, 2006). The block design task tests spatial ability by putting a set of blocks together to match a given pattern.

Further support for the cerebellum's involvement in the learning of spatial task is shown in chess players. When comparing new chess players to more experienced chess players, functional magnetic resonance imaging reveals that there is more activity in the cerebellum of inexperienced players in a figure positioning task (Steinlin, 2007). This magnetic resonance imaging suggests that the cerebellum is involved in the learning process more than the process of recalling strategies from memory because only new players had this increased activity. This involvement of the cerebellum in learning strategies is supported by rodent studies that use the Morris water maze (Lalonde & Strazielle, 2003; Petrosini, 2007).

Rodents. The Morris water maze allows researchers unique ways to study learning in mice and rats (Petrosini *et al.*, 1996). The maze can be used to study the development of spatial strategies, spatial learning and spatial working memory (Lalonde & Strazielle, 2003; Petrosini, 2007). The animal is placed in a large circular tub (2-4 m) of water made opaque with the addition of dry milk or other methods. A small platform (5-15cm) is submerged in the tub and the animal must find this hidden platform to escape the water. In the spatial versions of this maze, no cues that are spatially contiguous with the goal are provided, thus the animals must use distal cues such as posters on the wall as spatial cues to triangulate the position of the goal.

Rat studies with hemicerebellectomized rats and rats with bilateral damage to the midline cerebellum tested in the Morris water maze show that the cerebellum is involved in the learning of spatial strategies but not the recall of spatial strategies already learned (Lalonde & Strazielle, 2003). Additionally, spatial learning deficits appear to be due, in particular, to deficits in learning the appropriate search strategies rather than in learning the spatial location of the platform. Rats that were allowed to learn the location of a platform pre lesion can learn a new location; thus location knowledge is not impaired. However if rats are not allowed to fully develop search strategies before lesioning, rats are unable to develop search strategies, beyond pre-operative levels, after lesioning.

To determine the exact role of the cerebellum in learning strategic components of the Morris water maze, Petrosini (2007) did an ingenious experiment taking advantage of the fact that rats can learn the location of the hidden platform in the Morris water maze from observing other rats. In her experiments, rats were allowed to learn by observing other rats in the Morris water maze who had either fully developed their searching strategies, or watched rats that had not yet learned the task strategies. Then rats were lesioned. If the observers saw the full behavior before cerebellar lesioning, the observer rats showed no deficits in maze completion. If rats were not allowed to observe the full development of searching strategies before lesioning, they never developed their searching strategy beyond that of the rat that they observed (Petrosini, 2007). This is evidence for cerebellar involvement in learning specific procedural elements required to gain spatial knowledge in the Morris water maze rather than a role in learning spatial knowledge itself. As Petrosini (2007) characterized it, the cerebellum is involved in

learning "how" not in learning "where". Another brain region, the hippocampus, is involved in learning where the hidden platform is located.

Numerous studies have shown that damage to the hippocampus prevents learning the location of a platform in the Morris water maze (Bast *et al.*, 2009). In contrast, to the cerebellum lesioned rats, rats with hippocampus lesion will learn to search for the platform and even develop strategies to locate the platform quickly. But they will never be as fast at locating the platform as unlesioned controls because they do not directly approach the platform using spatial knowledge. This can be exemplified by training rats with hippocampal lesions and controls to locate the platform in a room with moveable distal cues then performing a "probe" trial. In the probe trial, the cues are rotated 180degrees, and the platform is removed. Control rats will persistently search for the platform 180 degrees opposite the original platform location while hippocampal lesioned rats will attempt their search strategy (such as searching one foot from the edge of the pool wall) and will then swim randomly with no persistent search of the previously correct quadrant. Once a rat has learned how to locate a platform in the Morris water maze and is then given a cerebellar lesion, it will not exhibit problems learning a new spatial location in the Morris water maze and will preferentially search for the platform in the previously correct quadrant during probe trials (Bast *et al.*, 2009).

One reason for cerebellar related deficits in learning the Morris water maze is the possible interruption of vestibular cue processing. The vestibular system gives animals a sense of balance and spatial orientation. Vestibular inputs along with visual inputs allow animals to calculate angular movements to reach a specific goal location. Under normal conditions the cerebellum mediates vestibular input to the medial temporal lobe.

However, the lesions of the cerebellum may interrupt this pathway causing spatial learning deficits (Lalonde & Strazielle, 2003).

Birds. Several studies in birds have shown correlations between behaviors and morphological characteristics of the cerebellum. For example, tool use is associated with complex cerebellar foliation patterns and the increases in the size of these folds when compared with birds that do not use tools. Given that tool use requires both cognition and spatial function, experimental studies are needed to further investigate the cerebellum's role in both cognitive and spatial aspects of tool use (Iwaniuk *et al.*, 2006; 2007). In a previous study, it was found that zebra finches show deficits in spatial working memory after bilateral lesions to the medial cerebellar nuclei in a plus maze task. The plus maze is a four arm maze made like plus symbol (+). In Spence et al. (2009) each arm of the maze had a cup with seeds in it. Three of them were covered with parafilm, which only allowed the bird access to food in one cup. The goal of this task is for birds to use spatial knowledge to find the cup with available food and working memory to avoid re-entries into incorrect arms. Lesioned birds made significantly more errors in arm choice while searching for food. In addition, lesioned birds showed no difference in the time to complete the task between the first and last day of the study, while control birds had significantly shorter latencies to complete the task when comparing the first and last days of testing. This showed that lesions to the medial cerebellar nucleus of zebra finches slows learning of the spatial working memory in the plus maze (Spence *et al.*, 2009).

Estrogen and Repair of the Cerebellum

As reviewed above, damage to the cerebellum or alterations in morphology in humans, rodents, and birds are associated with deficits in a number of visual-spatial, spatial working memory, and spatial neglect types of task, suggesting a prominent role for the cerebellum in spatial learning. While damage to the cerebellum or disorders associated with morphological abnormalities can lead to such deficiencies, there is also evidence suggesting that estrogen treatment may improve such symptoms and withdrawal of estrogen may increase such symptoms. There are many pathways by which estrogen could result in such behavioral changes but to review them would be beyond the scope of this paper. Thus I touch only lightly on mechanisms below.

Estrogen and Neuroplasticity

Studies of estrogen's role in neuroprotection have mainly been done in the hippocampus as this learning and memory region is highly plastic. Thousands of studies have shown that estrogen is involved in normal functioning, neuroprotection, and repair of the central nervous system with the majority of these studies done in the hippocampus (Foy *et al.*, 2000). Evidence stems from studies that show a positive correlation between estrogen treatment and reduced chances of the development of neurodegenerative diseases (Steinlin, 2007). Furthermore, endogenous or exogenous estrogen reduces the size of traumatic brain injury (Spence *et al.*, 2009). In addition to the reduction of size of traumatic brain injury, there is upregulation of aromatase synthesis, the enzyme that catalyses the conversion of androgens to estrogen (Garcia-Segura *et al.*, 1998; Spence *et al.*, 2009), in the region of injury even in brain areas where levels of aromatase are normally very low (Spence *et al.*, 2009).

Although, studies of estrogen's role in cerebellar function are few compared to those in the hippocampus (Garcia-Segura *et al.*, 1999; Peterson *et al.*, 2001; Peterson *et al.*, 2004; Zurkovsky *et al.*, 2006; Soderstrom *et al.*, 2009). In the last 10 years we have learned a great deal; a number of studies now attest to the possible role of estrogen in neuroprotection in the cerebellum. For example, estrogen provides neuroprotection against ethanol withdrawal in mice. Ethanol withdrawal leads to apoptosis of Purkinje cells. However, following twenty weeks of exposure to ethanol, mice with estrogen treatment had significantly less loss of Purkinje cells when compared to mice without estrogen treatment (Jung *et al.*, 2005).

Not only does estrogen protect from damage due to ethanol withdrawal but it improves cognitive performance of postmenopausal women, and may retard normal loss of cognitive abilities during aging. A study testing the ability of women to recall information from paragraph length stories compared women whose ovarian function was suppressed with control women who had normal ovarian function. This study showed that women who received estrogen treatments compared to those that were given placebo performed better in a memory test (Foy *et al.*, 2000).

To highlight the role of estrogen in cerebellar neuroplasticity, it is ideal to have a very plastic cerebellum so that one can detect even modest levels of modulation. Furthermore, we are more likely to find that neuroplasticity results in behavioral recovery when this plasticity is significant. Estrogen-dependent neuroplasticity in the mammalian cerebellum seems to be less robust than that seen in avian cerebellum. Thus, zebra finches offer a good model organism to study estrogen dependent neuroplasticity because of the relatively high neurogenesis that occurs in normal adult songbirds

(Peterson *et al.*, 2004). Furthermore, because cerebellar morphology is similar across taxa we can compare results in zebra finches to that in other species. It has been confirmed that estrogen receptor β is present in the avian cerebellum as in mammals (Ball *et al.*, 1999). In addition, mammals Purkinje cells are capable of neurosteroidogenesis only during development; while the avian Purkinje cells are capable of neurosteroidogenesis throughout life (Sasahara *et al.*, 2007). Although there is little aromatase expression in the normal zebra finch cerebellum, (Ball *et al.*, 1999; Peterson *et al.*, 2007) aromatase is upregulated by injury (Spence *et al.*, 2009). In both the zebra finch and rat brain, blocking aromatase, and thus estrogen synthesis, results in greater damage at brain injury site (Garcia-Segura *et al.*, 1998; Saldanha *et al.*, 2004; Wynne & Saldanha, 2004; Ryan *et al.*, 2008; Spence *et al.*, 2009). An increase in aromatase expression is correlated and believed to be induced by reactive astrocytes as well as thought to prevent a secondary wave of neurodegeneration after brain injury (Garcia-Segura *et al.*, 1998; Ryan *et al.*, 2008). Furthermore, in zebra finches, it is known that aromatase inhibition increases reactive gliosis and increases the number of degenerating cells at lesion sites (Saldanha *et al.*, 2004; Ryan *et al.*, 2008).

The neural evidence for estrogen's neuroprotective role in the zebra finch cerebellum is supported by behavioral outcomes in studies using the plus maze. In the plus maze, birds given cerebellar lesions and letrozole (blocking endogenous estrogen) plus estrogen replacement show a linear improvement by day in errors and latency to find the goal while birds with cerebellar lesions and letrozole without estrogen replacement do not. Thus, estrogen replacement aided in recovery of function after cerebellar lesion to the medial cerebellar nucleus (Spence *et al.*, 2009). While this study suggested estrogen

played a role in recovery of function, it did not specifically examine the role of estrogen in spatial learning. Thus, I now want to test the role of the avian cerebellum in spatial learning and examine whether estrogen improves any alterations in learning outcomes caused by lesions to the cerebellum. In my present study, I helped create a task to investigate the involvement of the cerebellum in spatial memory and learning without the need for pre-training. I investigate the role of estrogen in recovery of function using the aromatase blocker, letrozole, to prevent estrogen synthesis after mechanical lesioning of the cerebellum. In Experiment 1, I compared the performance of lesioned animals with endogenous estrogen, lesioned animals fed letrozole (no endogenous estrogen), and controls in an effort to better understand the cerebellum's role in spatial learning and estrogen's role in the cerebellum after injury. I predict lesions to the cerebellum will impair spatial function and that estrogen will aid in recovery of spatial abilities after cerebellar lesion. In Experiment 1, I administered letrozole orally which inhibits aromatase activity throughout the brain. To ensure that evidence for estrogen's role in recovery of spatial ability after cerebellar lesion were not confounded by lack of aromatase activity outside of the cerebellum in other areas of the brain, a second experiment was needed. To clarify that results seen in Experiment 1were from effects of letrozole action on cerebellar function, I performed a second experiment. In Experiment 2, I compare lesion animals, lesion animals fed letrozole, and lesion animals with letrozole administered directly into the cerebellum at the lesion site. I predict that in Experiment 2 each of the groups will perform similarly, meaning that results seen in Experiment 1 in birds fed letrozole are mostly from effects of aromatase inhibition in the

cerebellum and not in other brain areas because direct application to the cerebellum and systemic effects are the same.

Methods

Animals and Treatment

Male American Zebra finches (*Taeniopygia guttata*) of similar size and age were used. They were bred at University of Mississippi in the animal care center from initial stock obtained from multiple vendors. The subjects were all separated from the general breeding population 72 hours pre-surgery and housed together in cages (length 40.6cm, width 59.7cm, height 40.6cm) of no more than six birds. All procedures were approved by the University of Mississippi IACUC (protocol #07-015).

Experiment 1

Birds were randomly assigned to 3 different treatments: Les1 (Lesion + corn oil vehicle, n=6), Sys1 (Lesion + Systemic Letrozole, n=6) and Sham1 (Sham + vehicle, n=5)" groups. Letrozole is an aromatase inhibitor, an enzyme which produces estrogen. These groups allowed for comparison of birds with a lesion and blocked estrogen (Sys1), birds with lesions and normal endogenous estrogen levels (Les1) and Controls with normal estrogen and no lesion. Letrozole in corn oil (10mg/mL) or oil vehicle were administered by feeding 20ul of treatment daily. Animals were fed the treatment starting 72 hours pre-surgery and daily doses continued throughout training.

Experiment 2

Birds were randomly assigned to Les2 (Lesion + vehicle, n=5), Sys2 (Lesion + Systemic letrozole, n=6), and Loc2 (Lesion + Local letrozole, n=7) groups. Les2 and Sys2 groups were treated as above in Experiment 1. In Experiment 2, a group was added

that was given letrozole locally into the lesion site. At the lesion site 5µl of steroid suspension vehicle alone or containing 10% letrozole was released directly into the cerebellum to ascertain whether systemic administration of letrozole was similar to direct administration to the cerebellum. Similarity between these two groups would allow us to infer that systemic effects of letrozole in our spatial task were principally due to letrozole's effects at the cerebellum.

Surgery

Birds were weighed before surgery. Birds were briefly anesthetized using isoflourane then injected with 30 µl equithesin to initiate anesthesia and allow placement in a stereotaxic device (Kopf with small bird beak holder) outfitted to administer gas isoflurane via a tube inserted in the bird's beak below the beak holder. A small portion of the skull was removed to expose the cerebellum and central sinus. Using the intersection of central sinus between the cerebellum and the hemispheres as a zero point for lesion we made bilateral puncture lesions to the cerebellum with a 26 gage needle (Spence *et al.*, 2009). Experiment 1 lesion coordinates were L.M. ±1, R.C. -2.7, D.V -4.5. The needle was left in brain for one minute before being removed. For birds in the sham group the skull was only removed to the spongy bone layer. In Experiment 2, lesion methods were the same except that lesion coordinates were L.M. ±.7, R.C. -2.2, D.V -4.5 and for birds in the Sys2 and Let2 groups the needle was lowered into the brain and allowed to rest for a full minute. Then the needle was retracted 1mm and 5µl of steroid suspension vehicle (9mg NaCl, 5 mg sodium carboxymethylcellulose, 4µl polysorbate 80, 9µl benzyl alcohol in 1 ml distilled water) (Saldanha *et al.*, 2004) was injected into the brain over a period of one minute using (WPI, Micro4 Micro-syringe Pump Controller). Loc 2 birds

received a 10% letrozole solution in the steroid suspension vehicle. The needle was left in the brain for another two minutes then removed, and the laceration from the surgery was closed. Birds were allowed 48 hours to recover before behavioral testing began.

Apparatus

In order to test the spatial abilities of zebra finches, we built a clear cylinder (height 29.8 cm, diameter 30 cm) that sat on a ceramic tile that was heated by a hot plate. The hot plate was heated until the tile surface was at approximately 50°C to motivate the bird to escape. At temperatures below ≈ 46°C birds did not try to escape and above ≈ 56°C birds appeared stressed. The temperature of the hotplate floor was recorded before trials started and after trials were finished everyday (mean±standard error, 51.7±.2°C experiment 1, 50.6±.2°C experiment 2). The cylinder had a clear lid that could easily be opened by hand. A 5.5cm diameter hole cut 2.3 cm above the tile allowed the bird to escape from the cylinder once inside. The cylinder and hot plate were placed in the center of a zebra finch aviary cage (length 148.6cm, width 71.1cm, height 188.2cm). The bird's view of the room was limited by hanging a black cloth the full height of the 71.1cm sides but only half the height of the 148.6cm sides of the aviary to allow sufficient light into the test space. Three large cues were attached to the cloth walls of the aviary (blue square, orange triangle, and red circle), such that direct approach or avoidance of any one cue would not lead directly to the escape hole. Two perches spanned the width of the aviary at about 20 cm from the top of the aviary and 70 to 110 cm into the aviary. Previously it had been shown that the zebra finches do not visually recognize the hole cut in the clear arena as evidenced by initial trial and error search behavior on being placed in the arena.

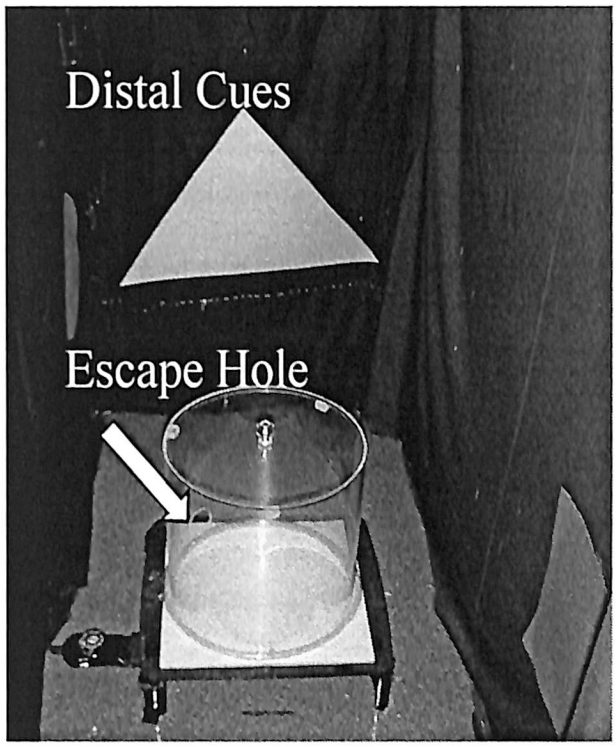


Figure1. The picture displays the testing apparatus with the 3 distal cues: circle, triangle, and square.

Behavioral testing

As birds were fed daily drug or vehicle treatments, they were taken from their home cage, placed in individual carry cages (length 31.1cm, width 15.9cm, height 15.2cm) and taken to the test room. Carrying cages were placed on a cart where the bird could not see into the testing aviary. Each bird was taken from their carrying cage and held in the hand of the experimenter with the hand in a lab coat pocket. The experimenter entered the aviary through a (45cm width, 162 cm height) and stood on markers on the floor that were ≈13 cm from the door and ≈15 cm from the apparatus. Given that the experimenter would be a large stable visual cue, it was important that this person remained in a stable position throughout testing to not confuse the birds. The experimenter opened the lid to the cylinder and placed the bird inside, then immediately

started a stop watch. When the bird escaped through the goal, the watch was stopped. Each bird was given two minutes to escape. If the bird did not escape in 2 min, the experimenter reached in the cylinder and coaxed the bird out of the escape hole and latency was recorded as 120s. The bird was allowed to remain in the aviary for one minute after completing a trail before being returned to a carrying cage. Birds typically sat on the available perches or in the far corner of the aviary during this rest period. We were careful not to move during this rest period and did not even look at birds during this period as this appeared to make the bird stressed and we wanted the rest period to be a positive reinforcer. Each trial was recorded with a digital camcorder and recordings were used to confirm latency and to track the pathway of the bird using an automated tracking program (dartfish company) to measure distance moved. (We are still in the process of making these distance measurements). Each bird had an inter-trial interval of about 10-20 minutes while we tested other birds. Each bird was then run three more trials for a total of 4 trials per day. We arbitrarily labeled quadrants of the maze NW, SW, SE, and NE and birds were released into center of these quadrants at North, South, East, and West accordingly. Order of release was random across trails with the constraint that we used all 4 quadrants each day. Due to time constraints, we could only test a maximum of 6 birds a day. Thus birds were divided into "batches". Each batch had two birds from each of the three treatment groups. Within batches, birds were tested in groups of three; order of groups tested and order of bird within a group was random. However, the order of birds within a group was conserved for each day. Birds in experiment 1 were run for eight days and birds in experiment 2 were run for seven days.

Probe trials

On the last day of training, we ran a "probe" trial. During this trial, the three distal visual cues were rotated 180 degrees and the experimenter moved 180 degrees to stand on the opposite side of the cylinder from that during training. The clear cylinder with an escape hole was replaced with an identical cylinder that had no escape hole. Probe trails were run to determine if zebra finches used the distal cues to escape the apparatus. If zebra finches were using the visual distal cues for orientation then they should search for the escape hole 180 degrees from its original location (Figure 2). The time spent in each quadrant of the maze was determined watching the video playback on a large TV with the quadrants drawn on the screen. The probe trial was watched four times and the time spent in each quadrant recorded using a stopwatch turned off and on as the bird entered and exited the quadrant. The distance covered in each quadrant will be determined with dartfish as for regular trials.

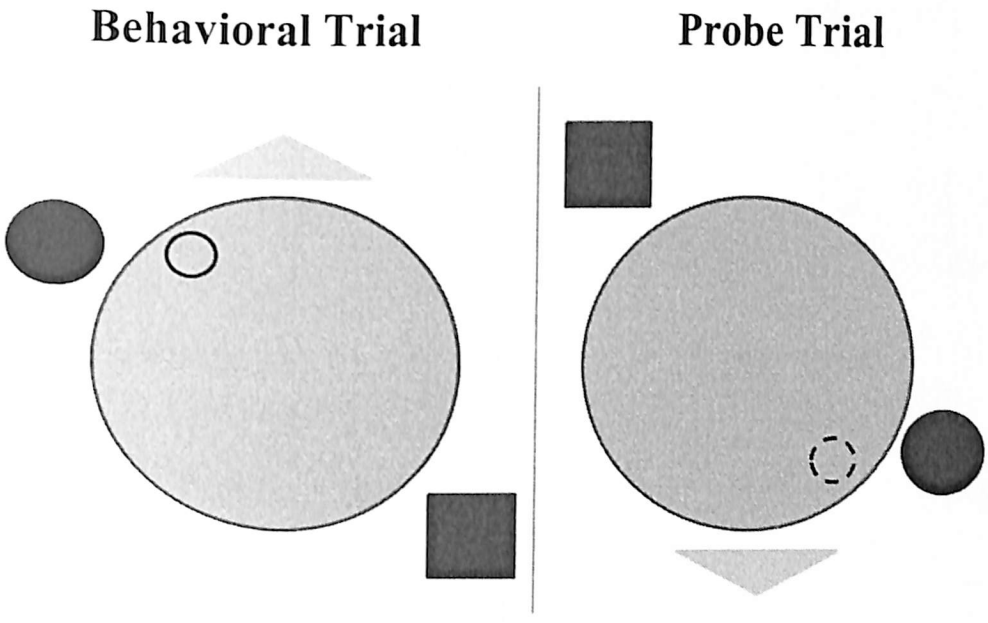


Figure 2. Top view of the apparatus in the behavioral and probe trials. In behavioral trial the cylinder contains a small escape hole (indicated by black circle). In probe trial the cues are rotated 180° from their original position and the cylinder with escape hole is replaced with a no escape cylinder. The position of the goal location in the probe trial is indicated by an arrow.

Histology

Birds were killed on the last day of testing by isoflourane overdose. The brain was then removed from the skull and fast frozen using dry ice. Brains were stored at -80°C. The cerebellum of each brain was sectioned at 40 μm and every section of the cerebellum was mounted on slides. Slides were stored at -80°C until staining. Brains were Nissl stained and lesion placement was confirmed using (Imager.M1, Carl Zeiss) using objectives 1.5x to 5x with reference to a pigeon atlas (Karten & Hodos, 1967).

Analysis

Data from both experiments were analyzed using STATVIEW 4.0 (SAS Inst.) on Macintosh OS9. The four trials run daily for each bird were averaged. Latency to escape was compared using repeated measures analysis of variance (ANOVA) followed by a t-test using Bonferroni correction for multiple t-test to compare treatment groups if the

ANOVA was significant. Repeated measures ANOVA was used to compare treatment groups during the probe trail and planned comparisons were used to investigate preference for the quadrant indicated by visual cues for each treatment group. The level of significance was $P<0.05$ for all comparisons. In Experiment 1, the goal was to have six birds for each group, however, one bird from the Sham 1 group died during testing; this bird's data was excluded from analysis. In Experiment 2, the goal was to have seven birds in each group but two birds died during the first three days of the experiment from surgery and one bird's foot was smashed during testing. These three birds' data were excluded from analysis.

Results and Discussion

Experiment 1

I found a significant difference between experimental groups ($F_{2,14}$=p<0.006). We used Bonferroni corrected post-hoc t-test to determine which groups differed. There was no difference in performance between Sham1 animals and Les1 animals nor between Les1 and Sys1 differ. However, Sys1 birds were slower to escape than Sham1 birds (p=0.005, to be significant with Bonferroni correction p<0.016). All of the groups did show some improvement in performance across days ($F_{7,98}$ =37.27, p<0.0001). There was no interaction effect.

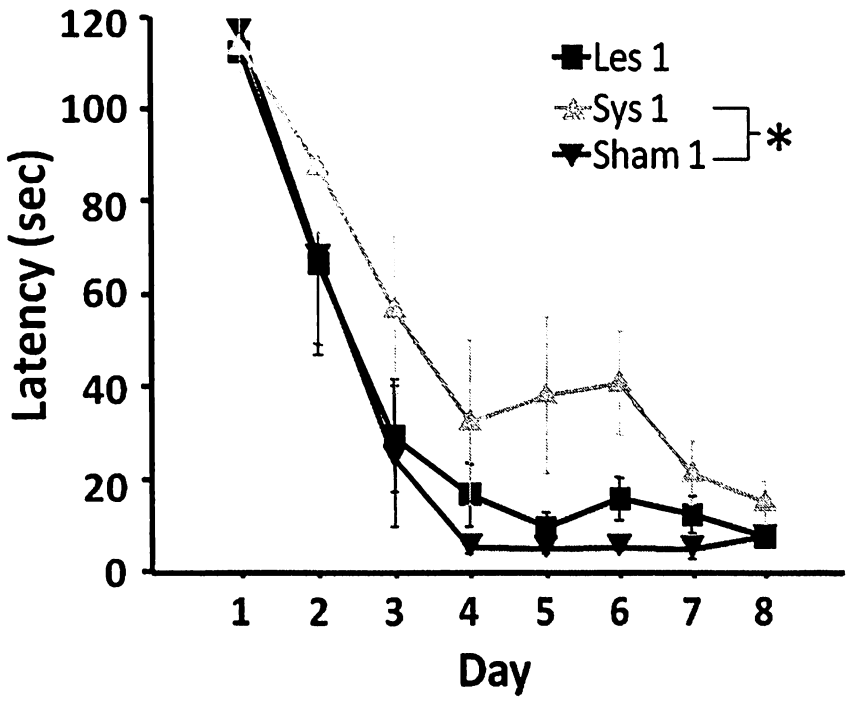


Figure 3. Shows the results of Experiment1. There was no difference in Les1 and Sham1 groups. There was also no difference between Sys1 and Les1 groups. Sham1 had a significantly shorter latency of escape than Sys1 as indicated by *.

In Experiment 1 probe trials, there was no difference between groups and no interactions. There was a significant effect of quadrant preference ($F_{3,39}$=23.79, p<0.0001). Each of the groups showed a preference for the quadrant 180° opposite the original goal location as compared to the other quadrants as measured by a planned comparison test (F_1=36.84, p<0.0001). These indicate that birds did not use olfactory cues or visual cues other that than the four cues that were rotated in the probe trails: the square, circle, triangle and the bird handler (Figure 4).

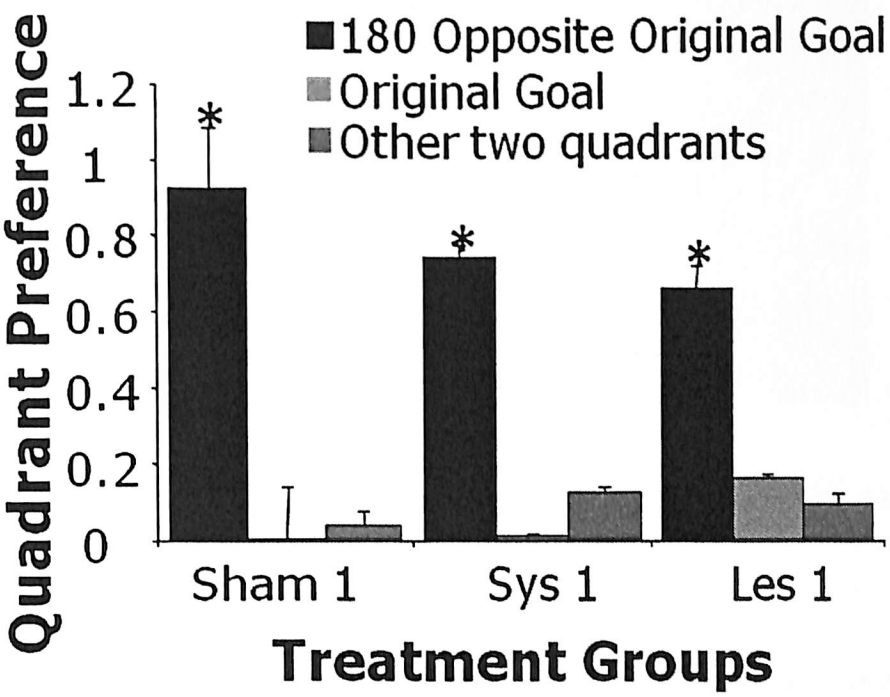


Figure 4. Each group showed a significant preference for the quadrant 180° opposite the original location. (Significant differences are indicated by *)

Lesion Confirmation. I examined nissl stained brains to confirm lesion placements. Lesions did not always directly hit the lateral deep cerebellar nucleus but did damage neuronal connections and the overlying folia of the lateral deep cerebellar nucleus (Figure 5).

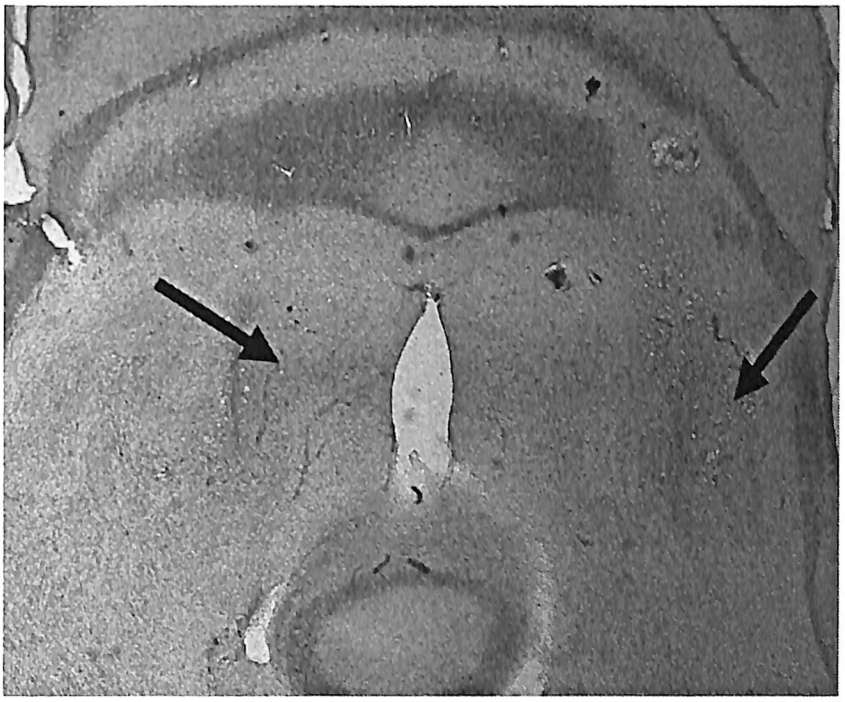


Figure 5. Shows brain tissue after nissl staining indicating exemplary placement of lesions. Black arrows point to the lesion site.

Because birds with lesions that had endogenous estrogen did not perform differently than controls, but birds with lesions and blocked aromatase performed significantly worse than controls, I concluded that by inhibiting aromatase production the affects of cerebellar lesions are intensified. Because I did not see differences between controls and the lesion group not treated with letrozole, one could suggest that the cerebellum is not involved in spatial learning. Instead, we suggest that the presence of endogenous estrogen in the avian brain in the lesion group was sufficient to reduce the

effects of lesions to control levels. While, birds without endogenous E were impaired compared to controls. However, since letrozole in Sys1 was administered orally, which means that aromatase was inhibited throughout the brain, data collected reflected the effects of the lack of aromatase activity throughout the brain. To ensure that impairments seen in Sys 1 are from lesioning the cerebellum with aromatase activity blocked and not the effects of letrozole on other brain areas, more specifically the hippocampus, another experiment was needed. The hippocampus has been reported to enrich place learning with moderate increases in estradiol levels (Zurkovsky *et al.*, 2006). From this one could infer that decreases in estrogen levels may impair spatial function, with this in mind I conducted Experiment 2 to ensure results in Experiment 1 were the result of impairments due to action of letrozole at the site of the cerebellar lesion.

Experiment 2

In Experiment 2, as expected, there were no significant differences across treatment groups (Figure 6). Each of the groups showed progress in latency to escape across days. Direct injection of letrozole into the lesion site did not significantly alter latency of escape when compared to estrogen administered orally. From the results of Experiment 2, I concluded that since there were no differences across groups for latency of escape that the effects seen in Experiment 1 are mostly the result of aromatase activity being inhibited by the presence of letrozole in the cerebellum.

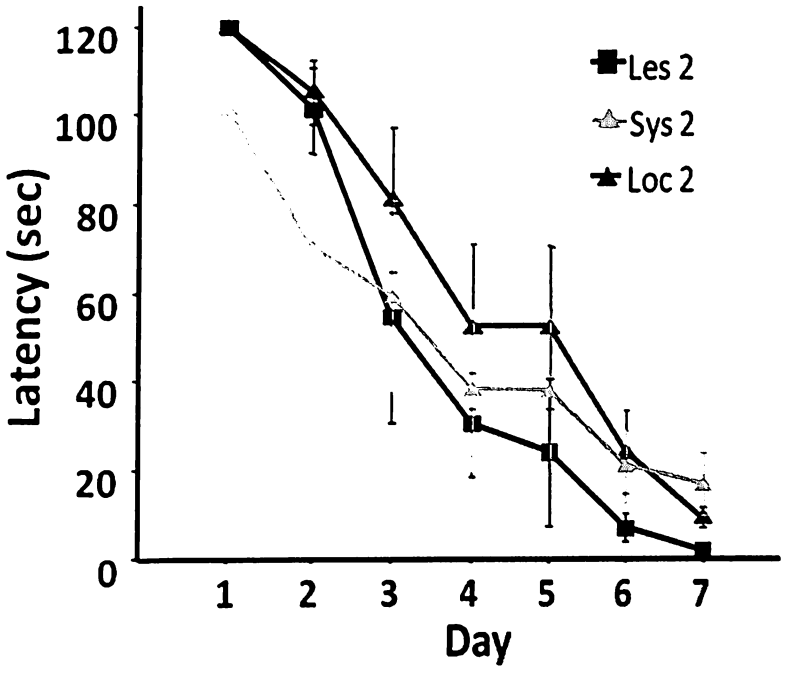


Figure 6. There was no significant difference between groups.

As expected, in probe trials there was no difference between groups and no interaction while there was a strong effect of quadrant ($F_{3,39}$=36.43, $p<0.0001$). Each group showed preference for the quadrant 180° opposite the original goal location in probe trials (planned comparisons, $F_1>105.3$, $p<0.0001$) (Figure 7). This points to the conclusion that each of the groups learned the location of the escape hole using the spatial cues provided. Thus, there was no deficit in learning the location of the goal.

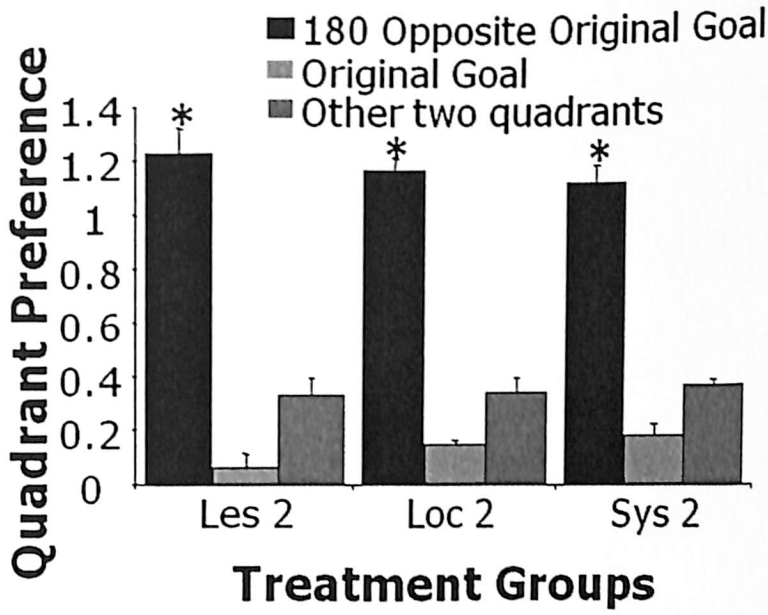


Figure 7. Each group showed preference for the quadrant 180° opposite the original goal (Significant differences are indicated by *).

Lesion Confirmation. I examined Nissl stained brains to confirm lesion placements. Lesions did not always directly strike the medial deep cerebellar nucleus in every experimental bird. However, in every bird lesions disrupted neural connections to each medial deep cerebellar nucleus and overlying folia of the cerebellum (Figure 8).

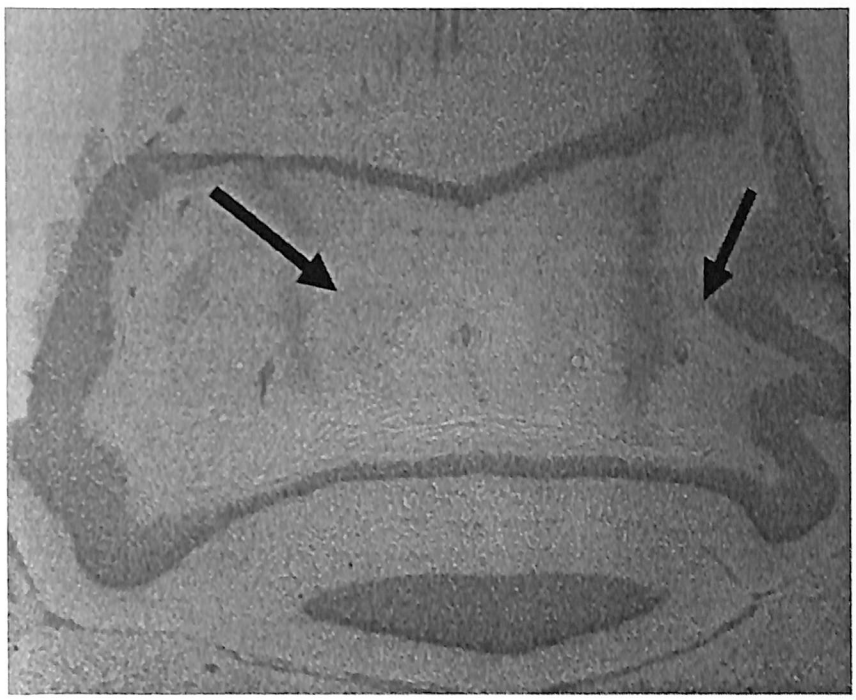


Figure 8. Displays an exemplary photomicrograph nissl stained brain tissue that shows the lesion to the medial deep cerebellar nucleus. Black arrows point to the postion of the lesion.

Conclusions

Our experiments are the first to test the role of estrogen in recovery of spatial learning in any species and the first to demonstrate that letrozole acting directly on the cerebellum may produce alterations in behavioral deficits caused by cerebellar lesions in zebra finches. Results of Experiment 1 clearly show that blocking estrogen synthesis impairs the normal course of recovery after lesions of the cerebellum. In fact, birds that have lesions of the cerebellum and endogenous estrogen were not significantly different from controls in performance on a spatial learning task. During probe trials all birds tracked the 180 degree rotation of the visual cues and searched for the escape hole in the quadrant 180 degrees opposite the original location. This suggests that the avian cerebellum is not necessary for recalling the location of a goal once it is learned, but instead is involved in acquisition of learning the goal location. This result is similar to what is seen in humans and in rodents (Lalonde & Strazielle, 2003; Petrosini, 2007).

The performance on the probe trials also suggest that I did not interfere with hippocampal function in my experiments. The type of spatial knowledge performed in the probe trial is known to involve the hippocampus in various species (Zurkovsky *et al.*, 2006; Bast *et al.*, 2009). Importantly, in zebra finches lesions of the hippocampus also impair their ability to locate a goal (Bast *et al.*, 2009). Furthermore, there is evidence that even moderate elevations in estradiol increase place learning in rats though the hippocampus (Zurkovsky *et al.*, 2006) suggesting that minor reductions in estrogen could cause deficits in spatial learning . Given that hippocampal-like spatial knowledge was intact in these zebra finches, I suggest that the actions of letrozole on the brain in Experiment 1, though systemic, were mainly affecting neuroplasticity at the lesion site.

At the very least, they were not unduly impairing hippocampal function. The results of Experiment 2 further support the assertion that the systemic letrozole treatment was similar to administration of letrozole directly to the lesion site. My results support and extend a previous study showing that lesions of the cerebellum interfere with spatial working memory and that estrogen improves recovery of function in that task (Spence *et al.*, 2009).

It was somewhat surprising that we did not see a deficit in learning the location of the escape when comparing birds with cerebellar lesions and endogenous estrogen to controls with sham lesions. While lack of estrogen decreased performance, the lesion itself was not sufficient to cause a deficit in this task. I believe this is due to the presence of endogenous estrogen improving repair at the lesion site. However, my results seem to contradict other studies showing that lesions of the cerebellum in zebra finches and in rodents do impair spatial abilities. My results likely differ from the previous zebra finch study because of differences in task difficulty or task demands. The plus maze used in Spence study (2009) required use of spatial working memory (remembering which alley had already been entered during a trial) while our task required spatial reference memory (knowing the location of a goal across trials). My results may suggest that the avian cerebellum is more important for working memory than reference memory as has been found under certain conditions in mice (Spence *et al.*, 2009). It may also be that it was easier for the zebra finches to acquire the spatial knowledge needed to solve the task in our study than in the Spence et al. (2009) study. My spatial cues were rather large (including a human only 2 ft from the clear cylinder) and there were only 4 prominent cues available while the Spence study used a blind to hide the experimenter and used

more dispersed and irregular objects already in the room (shelves, desk, pipes, etc) as cues. Thus, the procedural aspects of the task, the knowing "how" to acquire spatial knowledge, may have been reduced and deficits not seen until the lesion was paired with a reduction in estrogen.

Differences between our study and rat studies are more obvious. In the studies of spatial memory in rats, half of the cerebellum was removed. In our study, only a small lesion was produced. In addition, zebra finches have increased plasticity in the cerebellum compared to the rat cerebellum. Purkinje cells of the avian cerebellum are steroidogenic well into adulthood while steroidogenesis is seen only in the Purkinje cells of developing rat pups (Tsutsui *et al.*, 2003; Tsutsui *et al.*, 2006). Thus, zebra finches without blocked estrogen synthesis would be more likely to be producing estrogen in the cerebellum than would a rat. Further testing with larger lesions or a harder spatial task will be needed to understand if there are deficits in acquisition of a reference spatial task in cerebellar lesioned songbirds.

A previous zebra finch study showed impairments in spatial working memory after cerebellar lesions without blocking aromatase activity. However, the plus arm maze tests different types of spatial abilities than my escape maze and the format of the task required far more motor coordination than did my spatial. Birds had to walk down long alleys to obtain a food reward hidden in one of the four arms. In that study, some birds had gross motor impairments and fell over as they went down alleys while the animals in our study never fell. The animals in the plus maze also showed an increase in entries into non-goal arms demonstrating errors in spatial working memory as well as the differences in latency to complete the task partly do to motor deficits. Nonetheless, the lack of falls

in my birds vs. those in the plus maze study suggest differences in neural pathways being interrupted. Although both studies used similar lesion coordinates, a different steriotax was used and slightly different lesion positions were found. We have not yet completed lesion volume studies therefore we can not compare the volume of damage in our study to the volume of damage seen in the Spence paper.

Although my findings differ from previous literature showing that animals with damage to the cerebellum were able to learn a spatial task, they do show that estrogen improves learning after lesions to the cerebellum which is consistent with studies showing estrogen plays a role in neuroprotection or neural repair. Although this study mostly focuses on cerebellar function and does not include experiments to investigate the actual mechanism through which estrogen is acting, previous literature suggest some mechanisms. In zebra finches, estrogen has been shown to protect the brain against a secondary wave of neural degeneration that occurs after lesions when estrogen is not present (Ryan *et al.*, 2008). The secondary wave of degeneration would increase the amount of damage at the lesion, resulting in loss of more neural connections. We are in the process of examining the size of lesions with and without estrogen treatment to investigate this possibility. Another possibility is that estrogen added in recovery after injury by helping in restoring neural structure (Foy *et al.*, 2000). We have demonstrated that estrogen increases cerebellar neurogenesis and we are further investigating that process (unpublished data).

My results suggest that the cerebellum is involved in spatial learning though it takes a reduction in estrogen to bring out this defict. In addition, given that as all birds show a general improvement in the task, even those with letrozole, it appears

that the cerebellum is not essential to learning a spatial task but rather that lack of endogenous estrogen after cerebellar lesions slows learning. Thus estrogen either aids in recovery of some aspect of learning that has been lost by the lesion or estrogen improves the ability to learn the task over time. We do not know the exact avenue through which estrogen is improving spatial learning. More research is needed to know the exact mechanism through which estrogen is aiding recovery of function and in what specific aspects of spatial acquisition the cerebellum is involved. We plan to further investigate the involvement of the zebra finch cerebellum in spatial learning by increasing the complexity of the task and to study the injured brain tissue to help determine how estrogen is aiding in recovery of function in the cerebellum. We now have an excellent test of spatial reference memory and an exceptional model, the songbird, in which to test the role of estrogen from a behavioral and a mechanistic stand point.

References

Ball, G.F., Bernard, D.J., Foidart, A., Lakaye, B. & Balthazart, J. (1999) Steroid Sensitive Sites in the Avian Brain: Does the Distribution of the Estrogen Receptor α and β Types Provide Insight into Their Function? *Brain, Behavior and Evolution*, **54**, 28-40.

Bast, T., Wilson, I.A., Witter, M.P. & Morris, R.G.M. (2009) From Rapid Place Learning to Behavioral Performance: A Key Role for the Intermediate Hippocampus. *PLoS Biology*, **7**, 0730-0746.

Ebner, T. & Pasalar, S. (2008) Cerebellum Predicts the Future Motor State. *Cerebellum*, **7**, 583-588.

Foy, M.R., Henderson, V.W., Berger, T.W. & Thompson, R.F. (2000) Estrogen and Neural Plasticity. *Current Directions in Psychological Science*, **9**, 148-152.

Garcia-Segura, L.M., Wozniak, A., Azcoitia, I., Rodriguez, J.R., Hutchison, R.E. & Hutchison, J.B. (1998) Aromatase expression by astrocytes after brain injury: Implications for local estrogen formation in brain repair. *Neuroscience*, **89**, 567-578.

Garcia-Segura, L.M., Wozniak, A., Azcoitia, I., Rodriguez, J.R., Hutchison, R.E. & Hutchison, J.B. (1999) Aromatase expression by astrocytes after brain injury:

implications for local estrogen formation in brain repair. *Neuroscience*, **89**, 567-578.

Glickstein, M. & Doron, K. (2008) Cerebellum: Connections and Functions. *Cerebellum*, **7**, 589-594.

Hubley, A.M. & Jassal, S. (2006) Comparability of the Rey-Osterrieth and Modified Taylor Complex Figures Using Total Scores, Completion Times, and Construct Validation. *Journal of Clinical & Experimental Neuropsychology*, **28**, 1482-1497.

Iwaniuk, A.N., Hurd, P.L. & Wylie, D.R.W. (2006) Comparative morphology of the avian cerebellum: I. Degree of foliation. *Brain Behavior and Evolution*, **68**, 45-62.

Iwaniuk, A.N., Hurd, P.L. & Wylie, D.R.W. (2007) Comparative Morphology of the Avian Cerebellum: II. Size of Folia. *Brain Behavior and Evolution*, **69**, 196-219.

Jung, M.E., Gatch, M.B. & Simpkins, J.W. (2005) Estrogen neuroprotection against the neurotoxic effects of ethanol withdrawal: potential mechanisms. *Journal of Experimental Biology and Medicine*, **230**, 8-22.

Karten, H.J. & Hodos, W. (1967) *A Stereotaxic Atlas of the Brain of the Pigeon (Columba livia)*. Johns Hopkins Press, Baltimore.

Kelly, J.F., Bienias, J.L., Shah, A., Meeke, K.A., Schneider, J.A., Soriano, E. & Bennett, D.A. (2008) Levels of Estrogen Receptors Î± and Î² in Frontal Cortex of Patients with Alzheimer's Disease: Relationship to Mini-Mental State Examination Scores. *Current Alzheimer Research*, **5**, 45-51.

Lalonde, R. & Strazielle, C. (2003) The effects of cerebellar damage on maze learning in animals. *Cerebellum*, **2**, 300-309.

Liberman, J., Stewart, W., Seines, O. & Gordon, B. (1994) Rater agreement for the Rey-Osterrieth complex figure test. *Journal of Clinical Psychology*, **50**, 615-624.

Moore, F.L. & Evans, S.J. (1999) Steroid Hormones Use Non-Genomic Mechanisms to Control Brain Functions and Behaviors: A Review of Evidence. *Brain, Behavior & Evolution*, **54**, 41.

Peterson, R.S., Fernando, G., Day, L.B., Allen, T.A., Chapleau, J.D., Menjivar, J., Schlinger, B.A. & Lee, D.W. (2007) Aromatase expression and cell proliferation following injury of the adult zebra finch hippocampus. *Developmental Neurobiology*, **67**, 1867-1878.

Peterson, R.S., Lee, D.W., Fernando, G. & Schlinger, B.A. (2004) Radial glia express aromatase in the injured zebra finch brain. *The Journal of Comparative Neurology*, **475**, 261-269.

Peterson, R.S., Saldanha, C.J. & Schlinger, B.A. (2001) Rapid Upregulation of Aromatase mRNA and Protein Following Neural Injury in the Zebra Finch (Taeniopygia guttata). *Journal of Neuroendocrinology*, **13**, 317-323.

Petrosini, L. (2007) "Do what I do" and "Do how I do": Different components of imitative learning are mediated by different neural structures. *The Neuroscientist*, **13**, 335-350.

Petrosini, L., Molinari, M. & Dell'Anna, M.E. (1996) Cerebellar contribution to spatial event processing: Morris water maze and T-maze. *European Journal of Neuroscience*, **8**, 1882-1896.

Prabhakaran, V., Smith, J.A.L., Desmond, J.E., Glover, G.H. & Gabrieli, J.D.E. (1997) Neural Substrates of Fluid Reasoning: An fMRI Study of Neocortical Activation during Performance of the Raven's Progressive Matrices Test. *Cognitive Psychology*, **33**, 43-63.

Ryan, D.W., Bradley, J.W., David, J.B. & Colin, J.S. (2008) Inhibition of injury-induced glial aromatase reveals a wave of secondary degeneration in the songbird brain. *Glia*, **56**, 97-105.

Saldanha, C.J., Rohmann, K.N., Coomaralingam, L. & Wynne, R.D. (2004) Estrogen provision by reactive glia decreases apoptosis in the zebra finch (*Taeniopygia guttata*). *Journal of Neurobiology*, **64**, 192-201.

Sasahara, K., Shikimi, H., Haraguchi, S., Sakamoto, H., Honda, S.-i., Harada, N. & Tsutsui, K. (2007) Mode of Action and Functional Significance of Estrogen-Inducing Dendritic Growth, Spinogenesis, and Synaptogenesis in the Developing Purkinje Cell. *J. Neurosci.*, **27**, 7408-7417.

Soderstrom, I., Strand, M., Ingridsson, A.-C., Nasic, S. & Olsson, T. (2009) 17Î²-estradiol and enriched environment accelerate cognitive recovery after focal brain ischemia. *European Journal of Neuroscience*, **29**, 1215-1224.

Spence, R.D., Zhen, Y., White, S., Schlinger, B.A. & Day, L.B. (2009) Recovery of motor and cognitive function after cerebellar lesions in a songbird role of estrogens. *European Journal of Neuroscience*, **29**, 1225-1234.

Steinlin, M. (2007) The cerebellum in cognitive processes: Supporting studies in children. *Cerebellum*, **6**, 237-241.

Tsutsui, K., Hirotaka, S. & Ukena, K. (2003) Biosynthesis and action of neurosteroids in the cerebellar Purkinje neuron. *Steroid Biochemistry and Molecular Biology*, **85**, 311-321.

Tsutsui, K., Matsunaga, M., Miyabara, H. & Ukena, K. (2006) Neurosteroid biosynthesis in the quail brain: A review. *Journal of Experimental Zoology*, **305A**, 733-742.

Wynne, R.D. & Saldanha, C.J. (2004) Glial Aromatization Decreases Neural Injury in the Zebra Finch (Taeniopygia guttata): Influence on Apoptosis. *Journal of Neuroendocrinology*, **16**, 676-683.

Zurkovsky, L., Brown, S.L. & Korol, D.L. (2006) Estrogen modulates place learning through estrogen receptors in the hippocampus. *Neurobiology of Learning and Memory*, **86**, 336-343.