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Development of new methods to investigate the role of the avian hippocampus in memory formation in brown-headed cowbirds

Madeleine IR Brodbeck, *The University of Western Ontario*

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Abstract

The hippocampus is known to participate in a variety of cognitive functions in humans, non-human primates, rodents, and birds. A great deal of previous research on the avian hippocampus has examined its role in spatial memory. A question regarding avian hippocampal function that remains unanswered is, what is its role in encoding, retention, and retrieval of spatial information? To answer this question I, 1) describe spatial and non-spatial versions of a touchscreen task and my attempts to determine if the spatial version of the task is hippocampus dependent, and 2) describe the development of an implantable cryoloop which can be used to reversibly deactivate the avian hippocampus. I found that cryoloops are a successful technique for use in birds, and results suggest the task will be appropriate for the research question. This work lays the groundwork for future studies in studying hippocampus's function and the avian brain.

Keywords

Co-Authorship Statement

I performed the work in this thesis under the supervision of Dr. Scott MacDougall-Shackleton and Dr. David Sherry. Dr. Stephen Lomber designed and created our cryoloops, and closely advised us on the creation of the external cooling apparatus. He also advised us on the creation of the loops, cryoloop surgeries, temperature probes, and in conjunction with Dr. Stolzberg helped us attain thermal recording videos.

Acknowledgments

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Thank you to my mother, Isabelle Michaud, my father David Brodbeck, my brother, Jonathan Brodbeck, and my partner, Ross Richard for your endless and continued support. Thank you to the entire Rooster Teeth family, you have helped me through this more than you will ever know. Thank you Thomas Groulx, Laura Nanni, Kaitlyn King, Erin Spadafora, Jesse Merelaid and Chaney Finlayson, other close members of my family.

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Chapter 1

1   Introduction

1.1   Theories of Hippocampal Function

Investigating the function of the hippocampus may lead to a better understanding of

memory itself. Many different functions have been proposed for the hippocampus

including the consolidation of memory in general (Squire, 1992) and spatial memory in

particular (O'Keefe & Nadel, 1978). The hippocampus is widely studied in a variety of

different ways; it is such a popular structure that it even has an entire journal dedicated to

it: *Hippocampus*. Despite the fact that we have investigated the hippocampus's role for

decades, the hippocampus's exact function and role is not known. Additionally, the

hippocampus's role is not constant across species and tasks (Squire, 1992). Even when

focusing on spatial memory (considering there are other functions the hippocampus

serves, other than spatial memory), there is no scientific consensus on what type of

spatial information is processed, and how. What types of spatial information does the

hippocampus have a role in? When is the hippocampus important for the processing of

this information? Does the hippocampus physically store this information, or does it act

as an index to the neocortex?

1.1.1   The Standard Model

One of the first theories of hippocampus function was that the hippocampus acted as a

cognitive map (O'Keefe & Nadel, 1979). Since then, many different theories have been

proposed, tested, and critiqued, including the standard model of consolidation (Marr, 1971), the multiple trace theory (Nadel & Moscovitch, 1997), multiple storage sites hypothesis (Sutherland & Lehmann, 2011), and others (Kryukov, 2008; Vinogradova, 2001). The standard model of consolidation was outlined by Marr (1971) but first explicitly described by Squire, Cohen & Nadel (1984). The standard model states that memories are first represented in the hippocampus and undergo synaptic consolidation. With some time, memories are transferred to other parts of the brain through a process called systems consolidation. The standard model states that the hippocampus is important in the acquisition of memories, but less so in the retrieval of longer term memories because their representation is elsewhere in the neocortex.

Many have challenged the standard model, however, including some early proponents of the standard model (Nadel & Moscovitch, 1997; Nadel, Winocur, Ryan, & Moscovitch, 2007; Sutherland & Lehmann, 2011). Nadel & Moscovitch (1997) used cases of retrograde amnesia to point out weaknesses in the standard model. If retrograde amnesia due to hippocampal injury can extend to a lifetime, which it often can, then the hippocampus is not just temporarily storing memories. If memories are moved out of hippocampus, why would it take over 30 years? Systems consolidation should not take that long. In a critique of evidence for the standard model in rats, Sutherland & Lehman (2011), argued that there is not enough evidence to support the model. In the 34 different rodent studies that are reviewed, Sutherland & Lehman (2011) argued that only one shows actual evidence for this standard model. This one study demonstrated retrograde amnesia at a time interval which could imply systems consolidation (28 days), as the other studies in the review demonstrated RA at a time interval which was well within

cellular consolidation. The review questioned if systems consolidation has been demonstrated experimentally. One criticism is that in many hippocampal lesion studies, there is often still intact hippocampus. Additionally, almost all the studies they reviewed were well within the time of cellular consolidation, not necessarily systems consolidation. Cellular consolidation is a term for cellular and molecular changes, such as long term potentiation, that occur very soon after a new learning episode to strengthen a memory (Wixted & Cai, 2013). Taking these points into consideration, an argument can be made against the standard model.

1.1.2   Multiple Trace Theory

In Nadel & Moscovitch's (1997) critique of the standard consolidation model, they present their theory, multiple trace theory. The multiple trace theory states that information is encoded by hippocampus, and acts as an index, by means of a memory trace. Every time the memory is reactivated, a new memory trace and neuronal context is created. Over time, the hippocampus and the neocortex interact which each other and affect each other for these memories. This explains why old autobiographic memories are not always lost after hippocampal damage. Older memories have been revisited, and reactivated more often, meaning they have more traces, and are less likely to be affected unless there is complete hippocampal damage.

1.1.3   Multiple Storage Sites

Sutherland & Lehman (2011) also find problems with the multiple trace theory in their review. They claim that there is a simpler theory of hippocampal function to explain a

large amount of evidence. They agree with a multiple storage site hypothesis, that states that different memories are independently established in different networks in the brain. Some may be completely dependent on the hippocampus, and always will be and others may be elsewhere in the brain, completely independent of the hippocampus.

1.2   The avian hippocampus

The theories of hippocampal function described above all draw on data from research on the mammalian hippocampus. The avian hippocampus is a functional homologue of the mammalian hippocampus (Colombo & Broadbent, 2000). While anatomically very different in appearance, the hippocampus of birds and mammals serves many of the same functions (Colombo & Broadbent, 2000). Colombo and Broadbent (2000) demonstrate in their review of different results in mammalian and avian species that the avian hippocampus is important in similar spatial memory functions to the mammalian hippocampus. There is a great deal of research that demonstrates that the hippocampus is crucial in many avian species to spatial memory in a variety of different contexts, from food caching in black-capped chickadees, to spatial discrimination on a touchscreen in pigeons (Bingman & Jones, 1994; Brodbeck & Shettleworth, 1995; Colombo, Cawley, & Broadbent, 1997; Hampton & Shettleworth, 1996; Mayer, Watanabe, & Bischof, 2013; Sherry & Vaccarino, 1989; Watanabe, 1999, 2002; Watanabe & Bischof, 2004). From an evolutionary perspective, evidence suggests that in early vertebrate evolution the medial pallium in teleost fish was specialized for spatial memory. This function remained as the tetrapod brain evolved, leading to the hippocampus found today in both birds and mammals (Rodríguez et al., 2002).

Perhaps it is not clear exactly how the hippocampus works, however, it is quite clear that hippocampus has a role in spatial memory. There are many questions within each of the theories of hippocampal function outlined above; what kind of spatial information is important, and when is the hippocampus important are two themes in these theories that I will focus on. Here, I will mainly focus on avian work.

1.3   What kind of spatial information?

While it is clear that the hippocampus is important in the processing of spatial information, sometimes it is unclear *what* kind of spatial information is important. For example, there are clear performance deficits in animals with hippocampal damage on tasks that involve navigation in space (Colombo & Broadbent, 2000; Colombo, Broadbent, Taylor, & Frost, 2001; Morris, Schenk, Tweedie, & Jarrard, 1990; Watanabe & Bischof, 2004). There are often not clear performance deficits, however, in subjects with hippocampal damage on key peck operant tasks (Colombo & Broadbent, 2000; Mayer *et al.,* 2013; Watanabe, 1999). The difference between these tasks is that some are allocentric while others are egocentric. Allocentric tasks involve processing objects with respect to an external frame of reference, like searching in an arena. Egocentric tasks involve processing objects in reference to the self, like performance in an operant chamber. Nevertheless, some spatial deficits have been shown even in operant tasks following hippocampal damage (Good & Macphail, 1994; Watanabe, 1999, 2002). Thus, the relative importance of the avian hippocampus for allocentric and egocentric spatial memory tasks remains an open question.

1.4   When is spatial information important?

Another important component of hippocampal spatial processing is *when* the spatial information is important. Memory is first acquired, then stored, and later retrieved. Exactly when and where this happens in the brain is a question that remains unanswered. When focusing on spatial memory we know that the hippocampus has an important role. However, when the hippocampus is important in these phases is not always clear (Shiflett, Smulders, Benedict, & DeVoogd, 2003).

Watanabe (1999, 2002) hypothesized that for egocentric tasks in pigeons, the hippocampus is important in the acquisition of spatial information, but less important in retention. Watanabe (1999) trained pigeons either on a spatial or colour discrimination task. Once animals were trained, half of the subjects had their hippocampus aspirated. Watanabe found that lesioned birds and non-lesioned birds performed similarly on both the colour and spatial discriminations. In a second experiment, with a new group of subjects, he attempted training birds on the spatial discrimination following the lesion. The hippocampal-lesioned birds could acquire the colour discrimination, but not the spatial task. From this he concluded that the avian hippocampus is important in the acquisition of the spatial task. Watanabe (2002) later performed another similar experiment. Pigeons had two tasks, one a purely spatial discrimination, the other spatial with an added colour cue. Each time pigeons reached criterion for a certain location of key, the correct key was switched. For the spatial task with a colour cue, pigeons had a specific colour assigned to demonstrate which location was correct. Once animals were trained, they were hippocampally lesioned by aspiration. Animals in the spatial only task

group were slower to acquire the correct location. Those in the spatial task group with the colour cue added had no disturbance to their performance. From this, Watanabe again concluded the avian hippocampus is important in the acquisition of the spatial task.

For zebra finches in an allocentric task, the hippocampus was important in both the acquisition and retention of spatial information (Watanabe & Bischof, 2004). The finches were trained to forage for food amongst four feeders along the floor of an aviary. There were five groups in total: acquisition, acquisition control, retention, and retention control, and an overall control group. The acquisition group was lesioned after habituation, and then put into the discrimination training. In discrimination training, only one feeder was baited with a total of four sessions. For the retention groups, birds were immediately placed onto discrimination training and then lesioned after four sessions. The retention group also had a single retraining session where performance could be measured. Compared to the controls, lesion groups performed significantly worse in both conditions. From these results, the experimenters concluded that hippocampus is important in both acquisition and retention of information.

Other studies with black-capped chickadees have shown that the hippocampus is important for short term retention but not long term retention (Shiflett et al., 2003). Chickadees were trained to retrieve food from 10 different feeders. In the spatial memory task, the feeder remained in the same spatial location and all feeders were the same colour. In the visual spatial task, the feeder remained in the same spatial location and was differently coloured than the other feeders. Birds were split into groups which performed the spatial task or the visual spatial task. The sequence of training and testing was the same for both groups. One of the 10 feeders was randomly baited and birds would search

for the correct feeder. After the rewards were found, birds would return to their home cages, and the same feeder was baited. This repeated five times, for a total of five trials. One these five trials were completed, birds waited for one minute in their home cages and allowed to make a final choice. In their first experiment, birds received infusions of lidocaine through two cannulae implanted bilaterally over the hippocampus, which temporarily inactivated the hippocampus, before being placed in the arena, and then tested. In experiment two, only the spatial task was used. Birds were placed into a long term (3 h) or short term (15 min) retention group. Here, after the animals performed five trials, and then waited in their cages for either 3 h, or 15 min. Lidocaine was then infused and birds were immediately placed back into the testing arena to evaluate their performance. In experiment one, spatial birds performed worse than visual-spatial birds. In experiment two, deficits were only found with the 15 minute, or short term delay. The authors concluded that the hippocampus is important for the acquisition of spatial memory. They also concluded that the hippocampus is important for retention of spatial memory with a short-term retention interval, but not important with a long-term retention interval. The experimenters mention that the memories might still be located in the hippocampus, but more robust, and less likely to be affected by the lidocaine infusion, as a three hour time interval is quite short in the case of systems consolidation.

Considering the variety of different results on the importance of hippocampus in different phases of memory formation, the question that I want to address is "What is the role of the avian hippocampus in encoding, retention and retrieval of spatial information?" In this thesis, I focus on the encoding, retention and retrieval of egocentric spatial information.

1.5   Encoding, retention and retrieval

Determining the role of the hippocampus in spatial memory encoding, retention and retrieval cannot be accomplished with traditional lesion studies (e.g, ablation, aspiration, neurotoxin administration). The limitation with most traditional methods is that they cause permanent damage. Once the hippocampus has been removed, or damaged, it cannot be restored. However, after a lesion or damage in the brain takes place, the brain may in some cases be able to adapt and use different brain areas to solve problems the damaged area was once been used for. This phenomenon is known as recovery of function. Methods using lesions may not actually reveal the normal role of an intact brain area. The types of questions that can be asked about the hippocampus are also limited when using lesions. For example, using a traditional lesion technique would not allow for a brain region to be working for encoding, off for retention, and working again for retrieval.

There are alternative methods to traditional lesion techniques. One of these is drugs such as lidocaine that can temporarily inactivate a brain region, with function returning after a period of time. However, it can be difficult to control the spread of injectable chemicals. A more precise way of achieving reversible inactivation is by cooling the brain. Originally outlined by Salsbury & Horel (1983), reversible inactivation by cooling has been developed and refined by Lomber and his colleagues (Lomber,1999; Lomber, Payne, & Horel, 1999). Cooling brain tissue does not stop action potentials, but stops post-synaptic neuronal communication (Lomber, 1999; Lomber et al., 1999) and offers a method of rapid and reversible brain inactivation with anatomical specificity.

1.6 A method of reversible brain deactivation: Cryoloops

Cryoloops are surgically implanted devices that can reversibly deactivate brain tissue using cooling. Cryoloops have a variety of advantages over techniques that cause permanent damage, as well as over those that do not cause permanent damage (Lomber, 1999). For one, cryoloops completely avoid recovery of function. Because most of the time the animal has an intact brain, it does not need to use alternative strategies to solve problems during most of its life. In this way, cooling cryoloops can be used to ask questions about a brain region's functionality without the concern that an animal is using a different brain area or strategy to solve the problems. Cryoloops also provide an advantage in statistical power in an experiment. With a lesion experiment, two groups are required: one sham lesion to act as a control, and the lesion, or experimental group. In the case of cryoloops, animals act as their own controls, as they have a completely functional brain area to compare to their own brain area when it is not working. Statistically, this makes for a more powerful technique such that studies require the use of fewer animals. Additionally, animals are compared to their own brain regions, and this minimizes any issues to do with individual differences between subjects in different experimental paradigms. For this same reason, cooling cryoloops is a more ethical technique of brain deactivation. By only needing half of the subjects that would be needed with a traditional lesion experiment, the number of animals being used in the experiment is immediately reduced, and thus becomes a more ethical technique. It also allows for simple verification

of new tasks. An animal with an implanted cryoloop can perform a variety of different tasks to test if they are dependent on a specific brain region. During an ablation lesion surgery, there is a chance of damaging non-target tissue, and sometimes unintended fatalities. With implantation of a cryoloop, there is only a small amount of tissue disturbance. One of the other advantages with this method is being able to ask new and different kind of questions, such as the one I have address in this thesis, namely how does the hippocampus participate in different stages of memory encoding, retention, and retrieval. There would not be a way to ask this question so easily, by deactivating and reactivating areas of the brain during a spatial working memory task within the same session.

While there are many advantages to using this method, there are also some disadvantages. Structures to be cooled cannot be deeply imbedded in the brain. Brain regions to be cooled must be located in a sulcus or, on the surface of the brain. Additionally, the animal is tethered while it is being cooled, as a surgically implanted loop is attached to an outside cooling apparatus via flexible tubing. This means that special consideration needs to be taken when designing behavioural tasks for animals to perform while tethered to the apparatus.

Cryoloops have been very effective in studies of primates and cats. Some other cooling studies have been performed in birds using the Peltier method, which cools tissue using current between different types of materials (Long & Fee, 2008), but cryoloops have never been implemented in birds. Considering the hippocampus lies on the dorsal surface of the brain in birds, cooling the avian hippocampus seems like a practical method of inactivation. A lot of memory work is performed with mammals, however, the

hippocampus in mammals is embedded deeper in the brain. Using cooling cryoloop in

mammals to temporarily deactivate the hippocampus would be a much more complicated

procedure than using cryoloops in birds. Considering the avian hippocampus is

homologous to the mammalian hippocampus, the implementation of cryoloops in birds

presents a unique opportunity to study spatial memory and hippocampus. In this thesis I

test the feasibility of using cryoloops to cool the avian hippocampus, specifically in

Brown-headed cowbirds (*Molothurs ater*).

1.7   Brown-headed cowbirds

There were numerous advantages to using Brown-headed cowbirds in my studies.

For one, Brown-headed cowbirds, especially females, have a relatively large

hippocampus (Sherry, Forbest, Khurgel, & Ivy, 1993). Because Brown-headed cowbirds

are brood parasites, females must keep track of host nests. Due to this part of their

ecological history, it is likely why their hippocampus is large, and a great deal of research

has investigated their spatial memory (Guigueno, MacDougall-Shackleton, & Sherry,

2015; Guigueno, Snow, Macdougall-Shackleton, & Sherry, 2014; Sherry et al., 1993).

Perhaps there are better animals suited to studying spatial memory, such as the black-

capped chickadee, however, they are much smaller and therefore, implementing

cryoloops would pose a much greater challenge. One of the problems with trying to

design cryoloops for birds is that they will have to be relatively small. Having a larger

head to operate under will make it easier to design cryoloops suitable for avian species.

Brown-headed cowbirds are also readily available in London Ontario, as they reside in

temperate to subtropical regions in North America (Henninger, 1906). We also know that

Brown-headed cowbirds will work on an operant touch screen apparatus (Guigueno et al., 2015), which is egocentric. In sum, Brown-headed cowbirds are an excellent model to investigate avian hippocampus's function in the encoding, retention and retrieval of spatial memory in a working memory touch screen task while using cryoloops.

1.8   Objectives

I had two goals in this thesis: 1) To implant cooling loops in an avian species, Brown-headed cowbirds, for the first time, to cool and temporarily deactivate hippocampus. 2) To develop an egocentric spatial memory task that is hippocampus dependent, can be used with mobility restraints, and that can be used in the future to assess memory formation, retention, and recall. These two goals were developed with the aim to lay the groundwork for future studies that could determine what phases of memory formation hippocampus is most important in an egocentric task using the cooling method (1) and a spatial task (2). Implanting loops in an avian species for the first time will allow for a variety of different and new questions to be answered. Using the cryoloops in a way to answer a question about spatial memory formation will allow for a deeper understanding of hippocampus and the brain in avian species.

Chapter 2

2   Methods

In order to achieve both of my goals, I performed two different experimental procedures. In the first section of this Chapter, I present the methods I performed to test cryoloops suitable for Brown-headed cowbirds. In the second section of this Chapter I present the methods I performed to validate a behavioural task that would work with the cooling and answer my long-term research question.

2.1   Cryoloops

Through this phase of my project, I attempted to implant cryoloops in Brown-headed cowbirds for the first time. We used an apparatus similar to that described by Lomber, Payne & Horel (1999). We attempted a variety of different anchoring strategies for the cryoloops in birds. The general procedure is described here, and our best procedure for anchoring is described later in Chapter 3. We also obtained temperature probe recordings to verify that our apparatus successfully cooled the brain, and to investigate the spread of the cooling. Through temperature probe recordings we also attempted to find the best orientation and position for the cryoloop.

2.1.1   Subjects

Brown-headed cowbirds were caught in January and February of 2016 near Long Point Bird Observatory, Long Point, Ontario, and in April of 2017 at Ruthven Park Natural Historic Site, Cayuga, Ontario. Weights were taken at the time of capture. During this

study birds lived in individual cages with a lighting schedule that changed between the

Spring (14 h light:10 h dark) and Winter (10.5 h light: 13.5 h dark). Food consisted of

premium budgie seed (Hagen) mixed with oyster shells and 2 different types of powdered

dry food: Mazuri Small Bird Maintenance Diet and Mazuri Insectivore Diet. Birds had *ad*

libidum food access. In total, three birds were used for cryoloop procedures.

2.1.2   Apparatus

The cooling apparatus consisted of two separate components, the external cooling and

pumping units (Figure 2.1), and the surgically implanted loop (Figure 2.2). The cryoloop

was permanently implanted and the external units are connected only during cooling

procedures. The surgically implanted probes, or the cryoloops, are made up of the

following components: stainless steel hypodermic tubing (the "cryoloop"), a

thermocouple (to record the temperature of the loop), a connector (to attach an outside

thermometer, and monitor the temperature of the loop) and extended plastic tubing (to be

attached to the rest of the apparatus) (Figure 2.2). The stainless steel hypodermic tubing

is bent and manipulated to fit over a specific brain area. In this case, all cooling probes

were designed with the purpose to cool the dorsal surface of the avian hippocampus.

The external apparatus' components consisted of a methanol bath, dry ice, an

Erlenmeyer flask, a rubber stopper, a low flow Fluid Metering Inc. pump (Syosset, NY),

a dry ice bucket, flexible plastic tubing and a variety of connectors (Figure 2.1). The flask

was used as a reservoir for the methanol, with input and output tubes coming through a

rubber stopper at the mouth of the flask. Methanol was drawn through the input tube with

a pump, to a dry ice bucket containing enough methanol to cover the tubing. Dry ice is

added to the bucket until desired temperature of the loop is reached. The methanol then runs to the cryoloop, and back to the reservoir through the output tube. An Omega HH-25TC digital thermometer (Norwalk, CT) is also attached to the cryoloop's thermometer connector.

When the subject's hippocampus is to be cooled, the probe, fixed to the subject's head, is connected to the rest of the apparatus. While monitoring the temperature of the loop with the thermometer, the flow rate of the pump can be manipulated to control this temperature (i.e., increasing the flow rate decreases the temperature of the loop more quickly, and decreasing the flow rate allows the loop to increase in temperature).
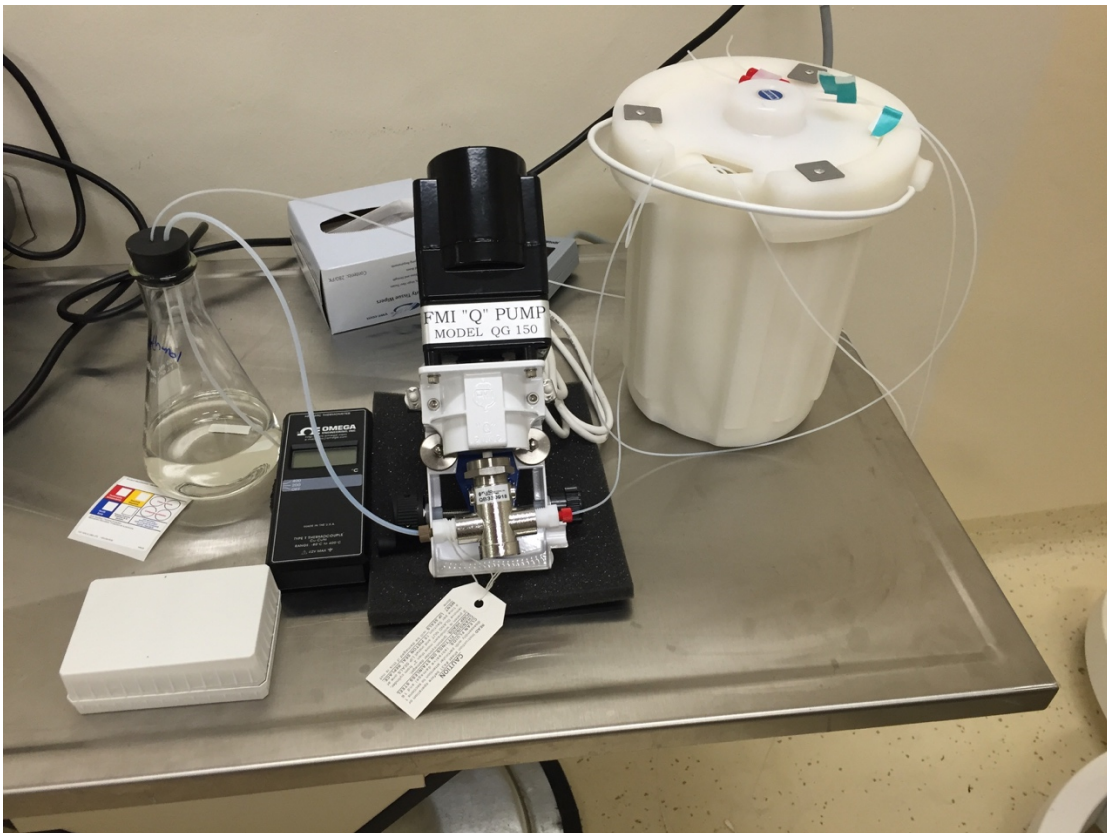
Figure 2.1. External cooling apparatus.
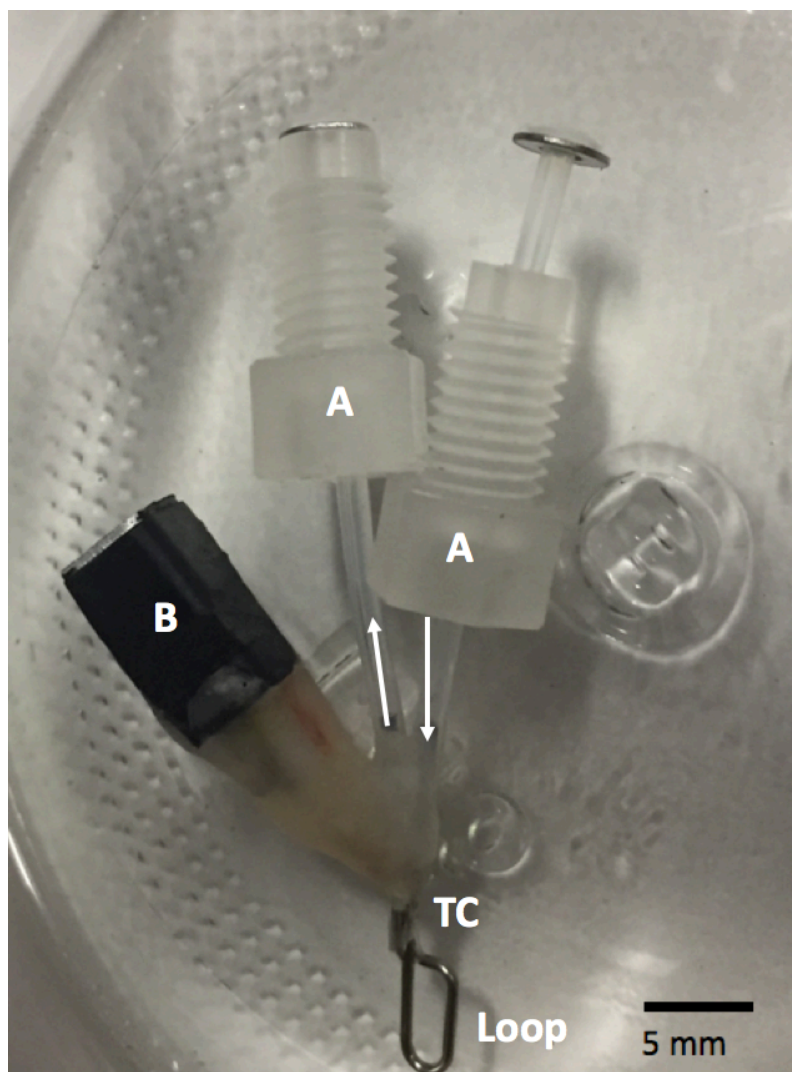
Figure 2.2. Cryoloop probe. A: Connectors which screw onto tubing that leads to external apparatus. B: Thermometer connector. TC: Thermocouple. Arrows denote flow of methanol.

2.1.3   Loop implantation

Loop implantation occurred while birds were under anesthesia. Birds were first given

0.01 mL Metacam (0.5% meloxicam) as an analgesic. Birds were anesthetized with 2-3%

isoflurane at a 2% flow rate of O_2. Birds were then moved to a stereotaxic frame for the

remainder of the surgery at the same percentage isoflurane and flow rate of O_2. The

feathers on the top of the head were wetted with ethanol to allow for a clean and clear

incision free of feathers. Birds were tested for a toe pinch reflex with forceps to

determine depth of anesthesia. Once the animal was fully anesthetized, the skin over the

incision was then washed with a sequence of 70% ethanol, Bacti-Stat (antimicrobial

soap), 70% ethanol, 2% chlorhexidine, and 70% ethanol. The skin was cut using surgical

iris scissors. Incisions were approximately 10 mm in length. The skin was opened and

moved to the sides to create a clear window for the craniotomy. Birds have pneumatized

bones, and the skull is in two layers with an air space between. Using a burr tip drill

multiple holes were drilled through the outer layer of skull, outlining the entire

craniotomy window. The holes were connected using a scalpel blade, and the outer skull

layer was removed using arch-tipped forceps. Depending on the procedure (see below)

the inner layer of the skull was then removed with forceps, or left in place.

For recovery surgeries, following the craniotomy the cryoloop was held in place

using a third arm and oriented to sit over the hippocampus. Saline soaked gel foam, cut

into 1mm x 1 mm cubes, was placed over top of the tissue to protect the tissue. Dental

acrylic was prepared and applied over the loop and intact skull. The acrylic was then

given at least 20 minutes to dry, and the arm clamp was released. The birds were removed from the stereotaxic, and placed in a recovery cage.

2.1.4   Temperature probe recordings

Two non-survival surgeries were conducted to investigate the spread of cooling throughout the brain tissue, and to validate the orientation of the loop. During both surgeries, birds were anaesthetized and fully functional cryoloops placed over the hippocampus. Temperature probe recordings were taken at different locations relative to the loop to determine the spread of the cooling throughout the tissue. At the end of the surgery, animals were euthanized with an overdose of isoflurane.

First, the cryoloop was attached to the outside cooling apparatus. During both surgeries, the procedure was identical to the procedure described above, in the surgical loop implantation section, up to the point of orienting the loop on the hippocampus.

2.1.4.1   Temperature probe recordings 1

The first temperature probe recording tested a cryoloop oriented horizontally, such that each of two parallel arms of the loop were placed above the left and right hippocampus, directly on top of the dura matter (Figure 2.3). During this temperature probe surgery, the inner layer of skull was also removed using a scalpel and forceps. The cryoloop was held in place using a manipulator attached to the stereotaxic frame and oriented directly over the hippocampus. The loop was placed in an orientation such that both arms of the loop contacted the brain tissue (i.e., one arm per hemisphere; Figure 2.3). The loop was connected to a thermometer that monitored the temperature of the loop. The temperature

probe recordings were then taken. A total of four different temperature probe locations were used: 0.5 mm lateral of right loop arm, 1 mm lateral of right loop arm, 2 mm lateral of right loop arm, and 0.5mm mediolateral of left loop arm. The probe was inserted into the tissue, and the cooling system was turned on. A tissue recording was taken once the probe's temperature reached a range of 2-5 °C. Once the recording was taken, the apparatus was turned off to allow the tissue to rewarm between temperature probe recordings. The probe was then lowered to different depths and locations and tissue temperature was recorded at each site.
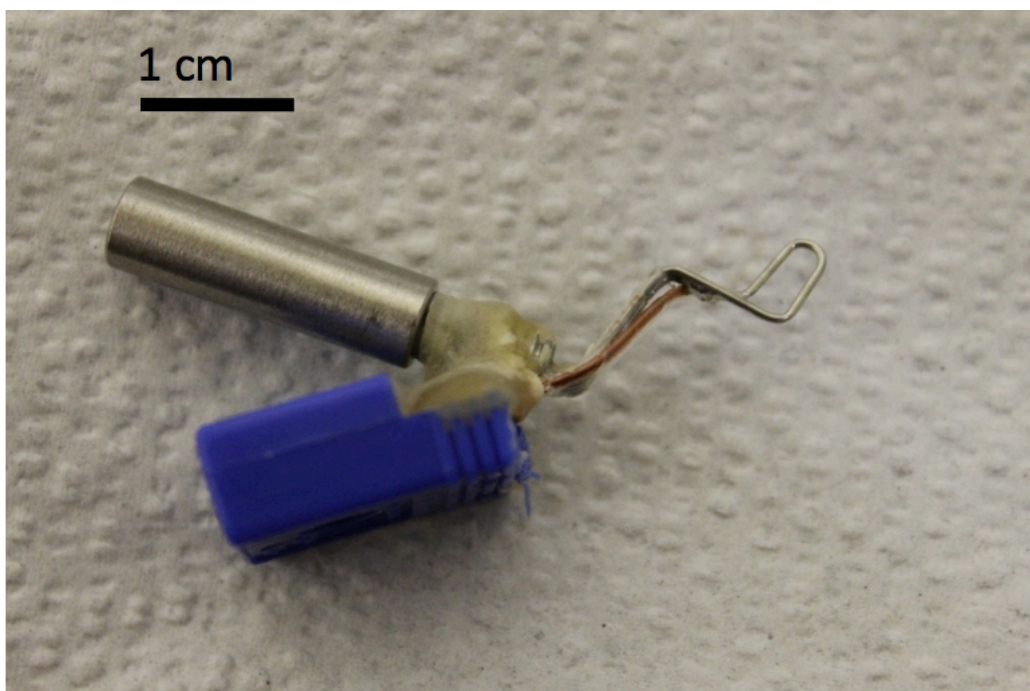
Figure 2.3. Orientation of the loop which places one arm above each hemisphere of the brain.

2.1.4.2   Temperature probe recordings 2

The second temperature probe recording tested a cryoloop oriented vertically, such that

one arm of the loop was placed directly above the midline of the brain between the left and right hippocampus, directly on top of the inner layer of the skull (Figure 2.4). During the craniotomy for the second temperature probe surgery, the inner layer of skull was not removed. After placement the loop was connected to a thermometer that monitored the temperature of the loop. To create a path for the temperature a hole was created in the inner layer of skull using a 26.5 gauge needle. Due to the bird's early mortality during the surgery, only one temperature probe location was chosen. Additionally, the loop was not turned off and back on to allow for rewarming, as the tissue would lack the ability to rewarm. The cooling loop's temperature was kept between 2-5 °C for each temperature probe recording. The location of the recording was in the left hemisphere, approximately 0.5mm away from the cooling loop. Temperature probe recordings of the tissue were taken at 10 different depths. The first was taken 0.5 mm deep into the tissue, and the next at 1 mm. After this depth, each recording was taken 1mm deeper, until a depth of 10 mm was reached.
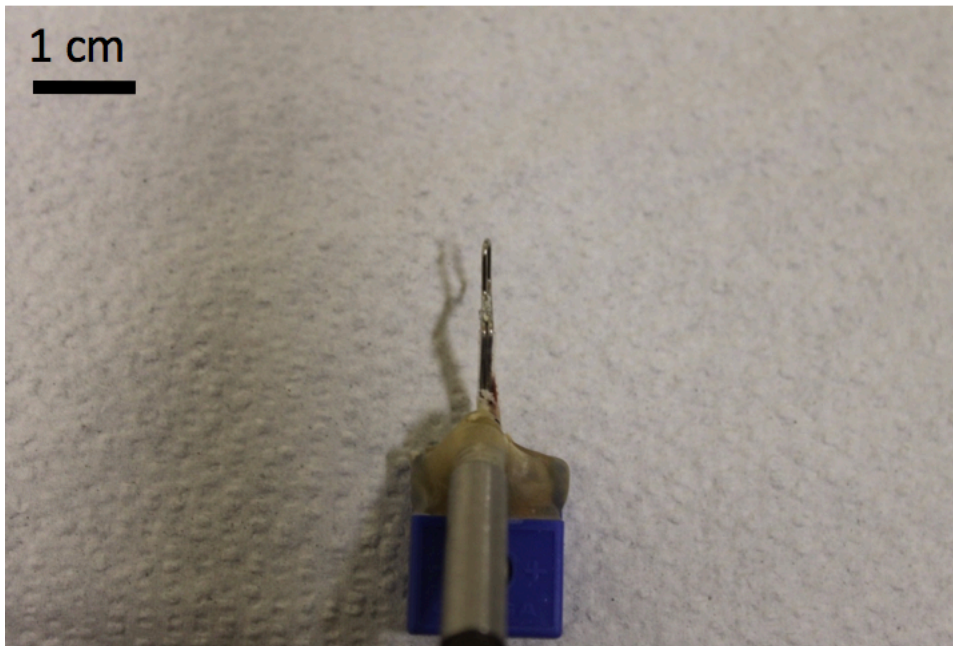
Figure 2.4. Orientation of the loop which places one arm down the midline of the brain.

2.1.4.3   Temperature probe histology

Brains were collected immediately from both birds following isoflurane overdose and soaked in paraformaldehyde for 4 days. Brains were then transferred to a solution of 30% sucrose, until fully saturated, and frozen with dry ice. Brains were stored in a -80 °C freezer until processed for histology. Brains were sliced coronally at 25 micrometer widths in a cryostat at -20 °C and thaw-mounted on to microscope slides. They were then stained for Nissl using thionin and cover slipped. Locations of the needle tracks were verified using a Leica microscope (Wetzlar, Germany).

2.1.5   Thermal imaging surgery

To further assess the extent and degree of cooling by the cryoloop I collected thermal image videos and photos in collaboration with S. Lomber and D. Stolzberg. Thermal

image videos also provided information on the rate of tissue cooling and rewarming. During this surgery, the bird was anaesthetized with a fully functional cryoloop placed over the hippocampus in a horizontal position, with each of two parallel arms of the loop placed above the left and right hippocampus, directly on top of the dura mater.

This procedure was performed during Temperature Probe 1 (2.1.4.1). Thermal imaging video was taken prior to temperature probe recordings, and after the loop was placed over the hippocampus. A thermal video camera (Flir model A320) was placed directly above the subject's head, with the loop and brain in frame. The video camera was set to record first with the entire apparatus off, to determine the starting temperature of the bird, the brain and the loop. The cooling apparatus was turned on and left on for 1 minute, and then it was turned off. The video continued to record for a total of 4 minutes.

2.2   Behavioural tasks

I created two different operant tasks to examine the role of the hippocampus in encoding, retention, and retrieval of spatial information: a spatial task and a colour task. Both tasks examine the contribution of the hippocampus to spatial and non-spatial memory. Based on prior research I predicted that the colour memory task would not require an intact hippocampus, and that the spatial memory task would require an intact hippocampus. To test these predictions, I trained four Brown-headed cowbirds on the tasks, lesioned the hippocampus using ibotenic acid, and then tested birds for an additional two weeks on the tasks to measure any change in performance (Figure 2.5). If there was no change in performance on the spatial task following lesion, then this task is not hippocampus dependent. After birds performed enough sessions to track performance, I collected their

brains, and verified lesion locations with histology.
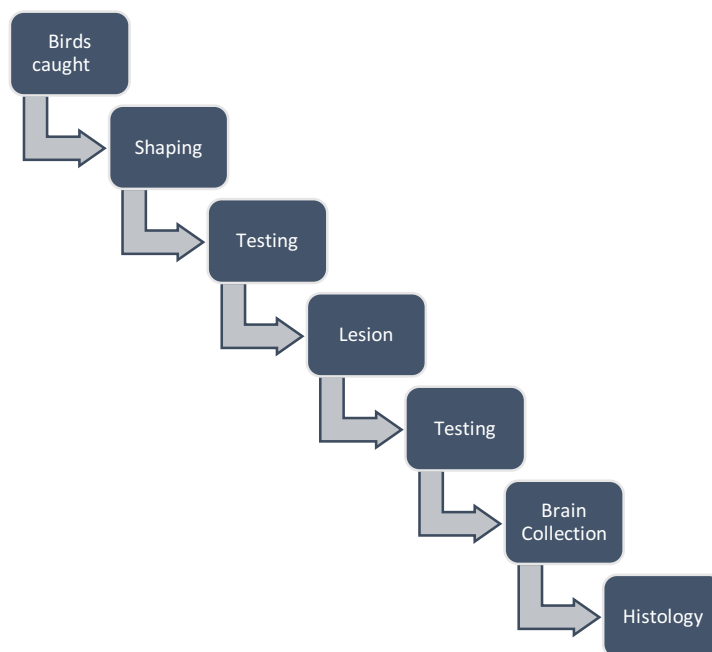
Figure 2.5 Overall timeline of the touchscreen task pilot experiment.

2.2.1   Subjects

See Section 2.1.1.

To ensure that birds would be motivated to work in the operant chambers, I placed birds on a food restriction schedule. I determined *ad libitum* weight once birds were in captivity on an *ad libitum* schedule for 3 weeks. The target weight for food-restricted birds was 85% of the bird's *ad libitum* weight. Birds were weighed daily before their sessions in the operant box. Here, it was determined if birds were over or under their target weight. If birds were overweight, they were given 2 grams fewer food than they received on the previous day. If birds were underweight, they were given 2 grams more food than they received on the previous day. If birds were at target weight, then they were

given the same amount of food they received the day prior. In total, four birds were used

in the lesion study.

2.2.2   Apparatus

Every operant box and corresponding computer (Figure 2.6) was enclosed in a sound

attenuating chamber (modified audiometric testing booth) (Figure 2.7). A ViewSonic

VG7106 computer monitor was placed on the outside of each sound attenuating chamber

to monitor the testing sessions without disturbing the birds. A total of three different

chambers were used during this experiment. Each sound attenuating chamber contained

an operant box designed for pigeons (33.5 cm x 31.0 cm l x 31.0 cm), the food hopper

control box, a touch screen control hub, a ViewSonic VG7106 computer monitor

(Walnut, CA) with an infra-red beam Elo touch screen (Milpitas, CA), and computer that

presented stimuli, monitored responses, and delivered contingencies (food reward or

lights out) (Figure 2.6). All testing was programmed using Experimentor software

(Version 2016.2.17.186). The computer monitor equipped with plastic protective sheeting

and touch screen frame formed one wall of the operant box directly in front of the food

hopper opening. The hopper was located on the floor three cm from the bottom center of

the touch screen. The hopper was powered and controlled by the food hopper control box,

and the touch screen was connected to the computer with the control hub.

Figure 2.6. Operant chamber, operant control box, touchscreen, touchscreen control hub, inside computer monitor, and computer tower.

Figure 2.7. Sound attenuating chamber with monitor to view performance.

2.2.3   Tasks

Both the spatial and colour memory tasks required birds to learn each day which stimulus

was reinforced. The reinforced stimulus (choices to location or colour) was consistent

within a day, but differed between testing days. Each daily session consisted of three

phases: an encoding phase during which birds learned which stimulus was reinforced, a

retention phase when no stimuli were presented, and a retrieval phase, which was identical to the encoding phase.

2.2.3.1   Shaping

Prior to the spatial and colour memory tasks, birds were shaped to use the operant chamber and peck symbols on the touch screen to obtain food reward (Figure 2.8). Shaping began with an initial day of habituation to the testing chamber, with food accessible around the hopper for 2 h. The following three days consisted of Basic Hopper Training 1, where birds would have access to the hopper every 5 s for 5 s. The next program was three days of Basic Hopper Training 2, where the hopper was immediately raised, and only lowered once the infrared sensor detected that the animal was eating from the hopper. This allowed me to track the number of times the birds fed from the hoppers. This program ran for 2 h each day, and birds had to reach a minimum criterion level of 100 trials per day to move on. Next was screen training. Here, one green circle (radius = 1.75 cm) was presented in the middle of the touch screen. Pecking the circle would give birds 5 s access to the food hopper. For the birds to move onto the next phase, a criterion of 100 trials three days in a row had to be reached.

The next phase of training presented birds with a choice of two items to peck. Items were presented on the lower half of the screen, and were 8 cm apart. Items were circles with a radius of 1 cm each. On a given session, pecks to one of the targets were reinforced (access to the food hopper), and the other target was not (lights out, no access to food hopper). From trial to trial the correct location of the target remained the same, but the colours changed randomly. The colours of the targets were: green, blue, orange,

purple or red. From day to day, the correct location of the target changed. Birds moved onto the next phase of training once they performed 70% correct or greater three days in a row (chance at 50%). The next phase of training presented birds with a choice of four items to peck. Items were presented on the lower half of the screen, and were 8 cm apart horizontally, and 3 cm apart vertically. All items were circles with a radius of 1 cm each. The colours of the targets were: green, blue, orange, purple or red. On a given session, responses to one of the items were reinforced (access to the food hopper), and responses to the other items were not (lights out, no access to food hopper). From day to day, the correct location of the target changed. Birds moved onto the next phase of training once they reach a criterion of 60% correct or greater three days in a row (chance at 25%), and had to experience each location as being correct at least once. Next, birds were moved to a program identical to the previous, with an introduction of the 20-minute retention phase. During these 20 minutes, birds were shown a blank white screen and could make no choices. This set of programs were named Spatial Choice. Birds were given three days on Spatial Choice.

After these three days, if the bird's performance had not dropped, birds were introduced to their first programs which relied on colour. This set of programs were named Colour Choice. Items were presented on the lower half of the screen, and were 8 cm apart horizontally, and 3 cm apart vertically. All items were 1.5 x 1.5 cm squares. Colours used were green, blue, orange, and purple. On a given session, responses to one of the targets, based on the target's colour was reinforced (access to the food hopper), and responses to the other coloured targets were not (lights out, no access to food hopper). The birds were placed on a schedule alternating between Colour and Spatial Choice

programs until each colour was presented to be correct at least once. Additionally, performance on the Colour Choice programs was at least 60% for three of these four programs (chance at 25%). Once birds reached this criterion, they moved onto the final versions of the spatial and colour tasks, described below.

Birds were placed on pseudorandom spatial and colour schedule (e.g. spatial task on day one, colour task on day two, colour again on day three etc.). This repeated for as long as needed, until the bird reached a correct average of 30% during the entirety of the task three sessions in a row for each task (chance performance would be 12.5%), and until each location and each colour had been shown to be correct at least once. Once the subjects reach this criterion, they will move onto surgery.
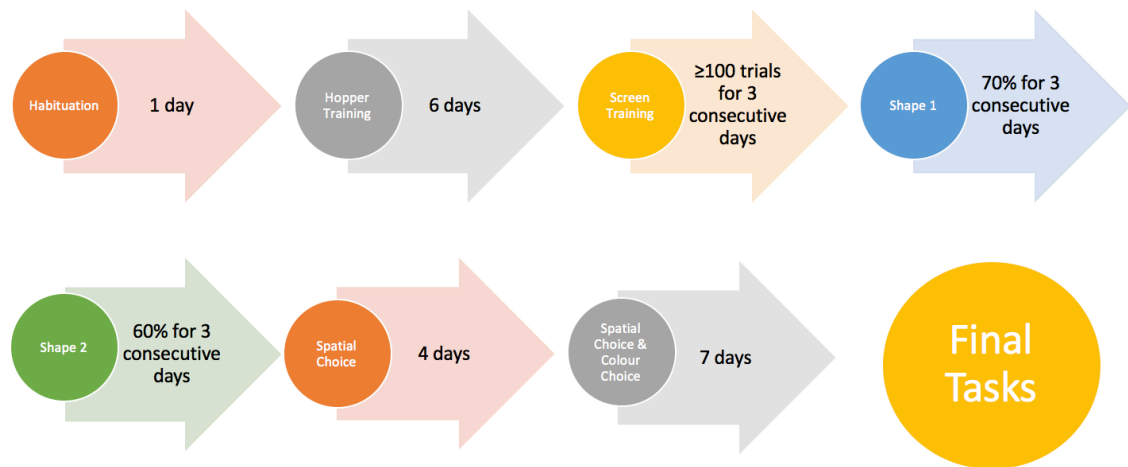
Figure 2.8. Shaping procedure.

2.2.3.2   Spatial task

During the spatial task, eight different targets would appear. On a given session, only responses to one target, in a specific spatial location, were reinforced with 5 s food access. Responses to the other seven locations resulted in the light in the chamber turning off, and no food access, for 5 s. From trial to trial, the colour of each target changed, but responses to the same spatial location remained reinforced. Colours used were green, blue, orange, purple, red, fushia, teal and light pink. This initial *encoding* phase lasted 20 minutes (Figure 2.9). Next, during a 20 minute *retention* phase, no targets were displayed on the screen, and the bird could make no choices. Next, during the *retrieval* phase, the bird needed to retrieve the correct location that it learned in the encoding phase (Figure 2.9). The retrieval phase consisted of trials identical to those in the encoding phase. Birds completed one session per day. The reinforced location choice changed randomly from session to session but was consistent across trials within a session. There was no fixed number of trials per session.
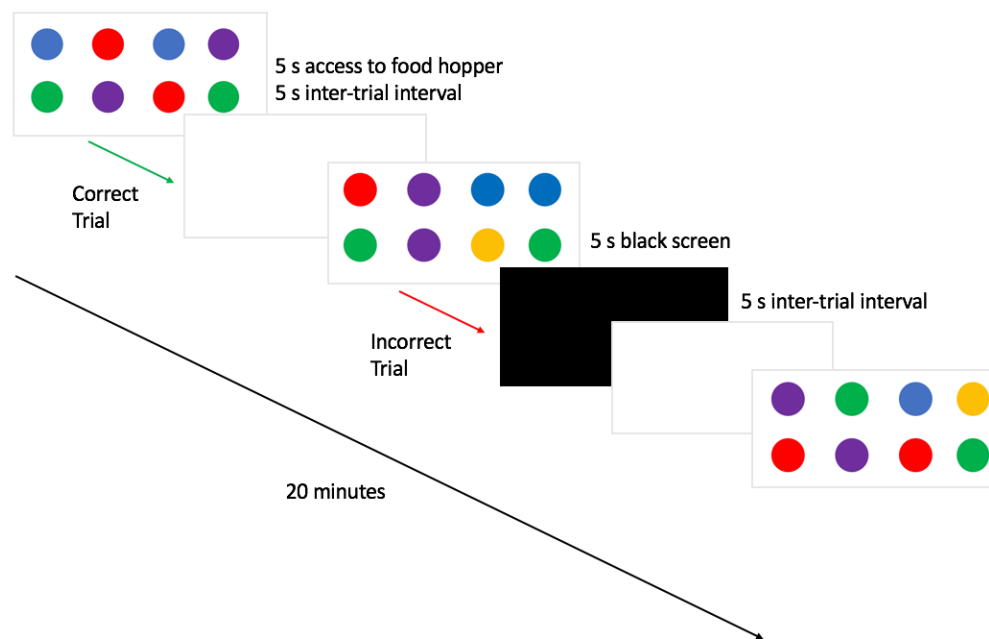
Figure 2.9. Encoding and retrieval phase progression for spatial task. Both phases are identical in progression. The overall progression of the task is encoding, then retention, and finally retrieval.

2.2.3.3　Colour task

During the colour task, 8 different targets would appear. On a given session, only responses to one target, of a specific colour, were reinforced with 5 s food access if the. Responses to the other seven colours resulted in the light in the chamber turning off, and no food access, for 5 s. From trial to trial, responses to the spatial location of each target changed, but the same colour remained reinforced. Colours used were green, blue, orange, purple, red, fuchsia, teal and light pink. This initial *encoding* phase lasted 20 minutes (Figure 2.10). Next, during a 20 minute *retention* phase, no targets were displayed on the screen, and the bird could make no choices. Next, during the *retrieval* phase, the bird needed to retrieve the correct location that it learned in the encoding phase

(Figure 2.10). The retrieval phase consisted of trials identical to those in the encoding

phase. Birds completed one session per day. The reinforced colour choice changed

randomly from session to session but was consistent across trials within a session. There

was no fixed number of trials per session.
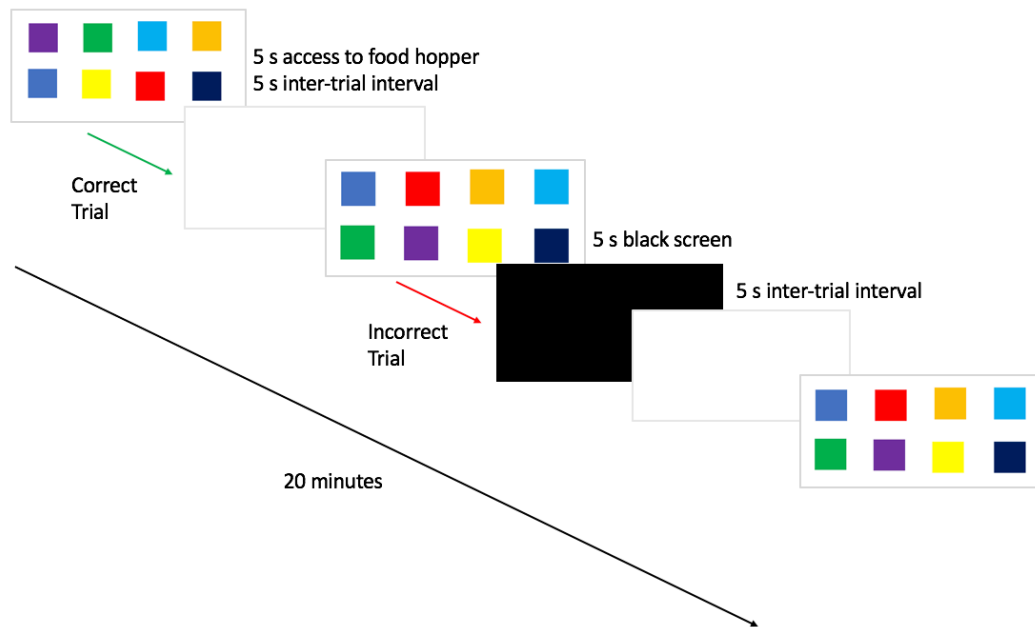
Figure 2.10. Encoding and retrieval phase progression for spatial task. Both phases are

identical in progression. The overall progression of the task is encoding, then retention,

and finally retrieval.

2.2.4   Lesion surgery

Once birds reached criterion in the eight item tasks I performed bilateral ibotenic acid

lesions of the hippocampus. Birds were first given 0.01 mL Metacam (0.5% meloxicam)

as an analgesic. Birds were anesthetized with 2-3% isoflurane at a 2% flow rate of O_2.

Birds were then moved to a stereotaxic frame for the remainder of the surgery at the same isoflurane dose and flow rate of O_2. When the bird was no longer responsive to a toe pinch the feathers on the top of the head were wetted with ethanol to allow for a clean and clear incision free of feathers. The skin over the incision was then cleaned and disinfected with a sequence of 70% ethanol, Bacti-Stat (antimicrobial soap), 70% ethanol, 2% chlorhexidine, and 70% ethanol and the incision (5 mm) was cut using surgical iris scissors. Next, using a mounted Dremel tool, four holes were drilled through the top layer of the skull. The central sinus at the junction of the cerebellum and two cerebral hemispheres and midline were both identified visually through the skull. Holes were drilled 1.5 mm and 3.5 mm anterior to the sinus, and 0.5 mm lateral from the midline for each hemisphere. Once all four holes were drilled, a needle was used to puncture each hole through the second layer of skull and the dura mater. A 1 µL Hamilton syringe mounted in a stereotaxic injector on the stereotaxic frame was manipulated to place the needle tip over the first hole and was then lowered into the tissue 2 mm deep, and brought back up 1 mm, to ensure a track for the acid to diffuse. Over a period of three minutes, 0.1 µL of ibotenic acid was slowly infused. This was repeated for the three other holes. Next, the skin was pulled close and mended with surgical adhesive (Vet Bond). Birds were removed from anesthesia and placed in a recovery cage with a heating lamp. Once birds were awake for a few minutes they were moved to their home cages. Birds were monitored every hour for the remainder of the day. During recovery, birds were closely monitored for health, and placed on an *ad libitum* food schedule. Birds were given 0.01 mL of metacam (0.5% meloxicam) daily during recovery. After three days of recovery, birds were placed back on the testing schedule in the operant boxes, and ran for a

minimum of 16 days (one day for each program version).

2.2.5   Histology

After at least two weeks, birds were anesthetized with isoflurane and perfused with saline

and paraformaldehyde. Brains were extracted and placed in a solution of

paraformaldehyde for one day. Brains were then transferred to a solution of 30% sucrose

until they were completely saturated. Brains were frozen with pulverized dry ice and

placed in a -80 °C freezer. When brains were ready for processing, they were sliced in a

Cryostat at -20 °C at 40 µm thickness. Brains were float mounted onto slides and stained

for Nissl with a thionin staining procedure.

Chapter 3

3   Results

3.1   Cryoloops

3.1.1   Loop implantation

A cryoloop was successfully installed in one subject (Figure 3.1). The best method for anchoring the cryoloop to the skull was by threading wires through four holes drilled around the outside of the craniotomy window (Figure 3.2). During prior pilot surgeries a variety of different techniques were attempted during cryoloop installation, this was the only method that resulted in secure attachment of the cryoloop. Following surgery the bird recovered and was able to perch and feed normally after a few hours. During recovery the bird was treated daily for three days with 0.01 mL metacam (0.5% meloxicam) as an analgesic. Despite the initial success of this installation, after 3 days the cryoloop and attachment became detached, and therefore the subject was immediately euthanized.

Figure 3.1. Subject with cryoloop successfully installed perching and alert.
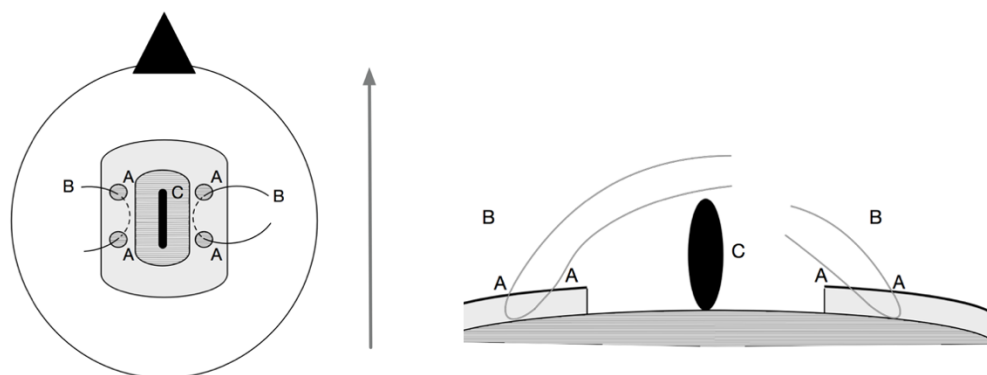
Figure 3.2. Anchoring method for attaching the cryoloop to the skull. Left Panel: Top view. Right Panel: Side view. The arrow denotes the orientation of the head. The light grey shaded area denotes the surgical opening with the outer layer of skull exposed. The striped area denotes the craniotomy with the first layer of skull removed, with the second layer of skull exposed. Four holes (A) were drilled through the top layer of the skull to allow two pieces of 32 gauge stainless steel wire (B) to be threaded through. One side of the wire was made to be longer than the other side. The right panel shows how the longer wires were bent over the cryoloop (C). Both sets of wires acted as anchors for the dental acrylic.

3.1.2   Temperature probe recordings

3.1.2.1   Temperature probe 1

 Cryoloops successfully cooled tissue when placed on the dura mater of an anesthetized bird. Temperature probe 1 recordings showed diffusion of the cooling throughout the tissue, with recordings taken further away from the probes being higher, and temperatures closer to the probes being lower (Figure 3.3). Significant cooling (20° C and below)

diffused past hippocampus, into pallial areas of the brain. Laterally, the cooling diffused past hippocampus as well, reaching parahippocampal areas.
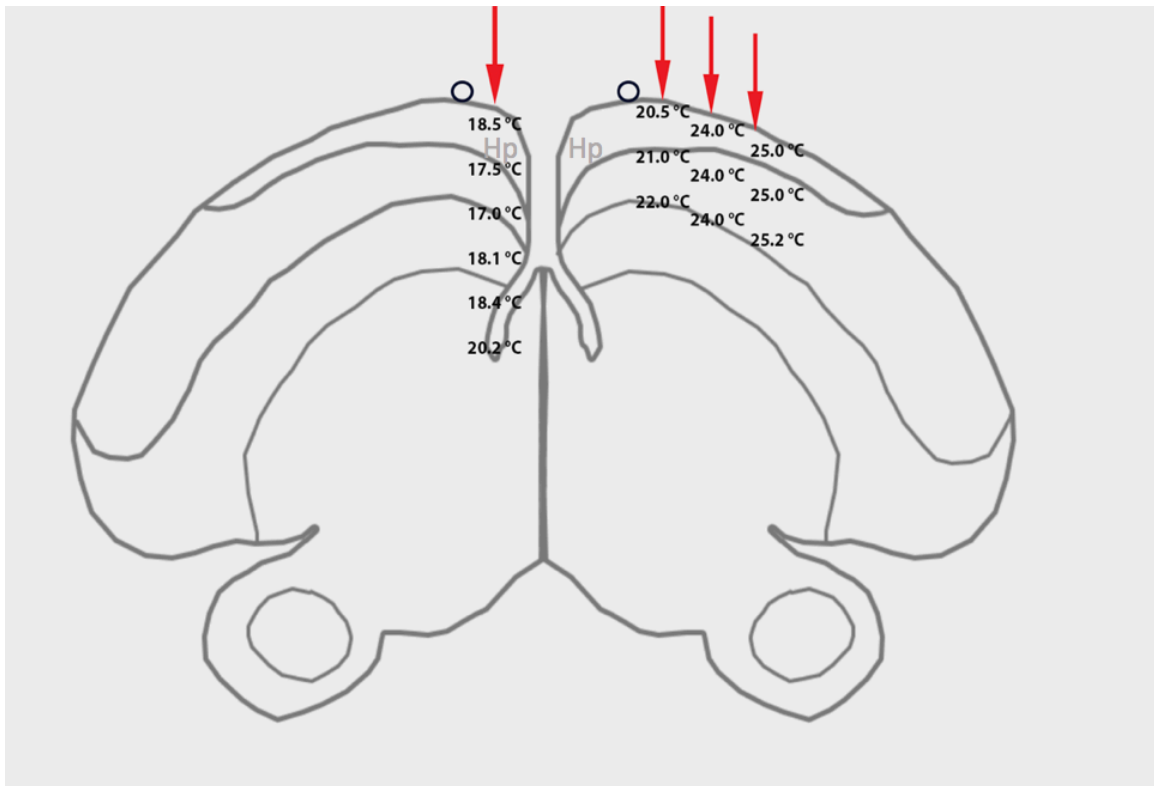
Figure 3.3. Temperature probe recordings overlaid on Brown-headed cowbird brain tracing (coronal; 1.5 mm anterior to sinus). Red arrows denote the location of the temperature probe recordings. Open circles denote the cryoloop location. Temperature recordings are 0.5 mm apart in depth. Hp indicates the location of the hippocampus.

3.1.2.2   Temperature Probe 2

Cryoloops successfully cooled tissue with one arm of the loop on top of the inner layer of skull. Temperature probe 2 also shows diffusion of the cooling throughout the tissue,

with recordings taken further away from the probes being higher, and temperatures closer

to the probes being lower (Figure 3.4). Temperature probe 2 showed that tissue was

cooled in the hippocampus. During temperature probe 2, significant cooling (20 °C) did

not diffuse into pallial regions of the brain.
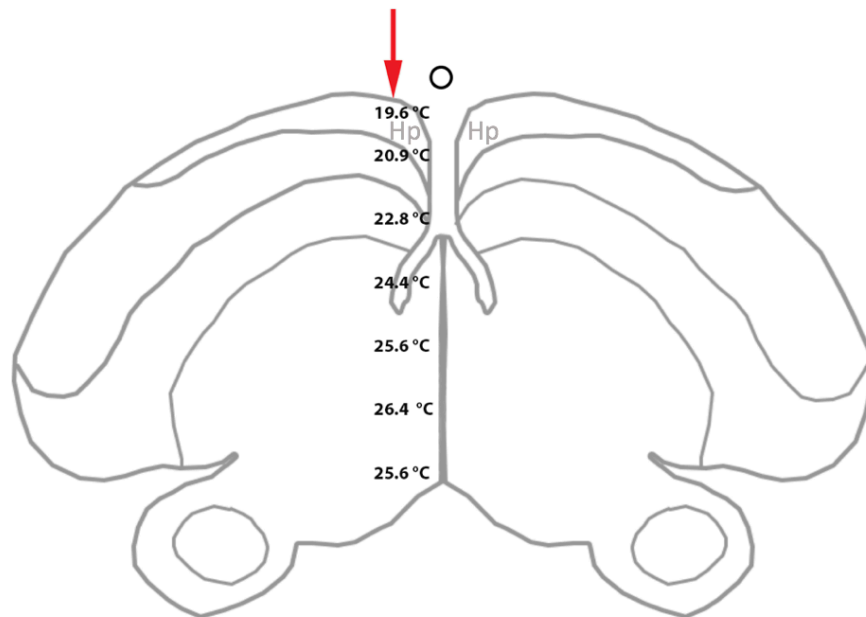
Figure 3.4. Temperature probe recordings. Temperature probe recordings overlaid on

Brown-headed cowbird brain tracing (coronal; 1.5mm anterior to sinus). The red arrow

denotes the location of the temperature probe recordings. The open circle denotes the

cryoloop location. Temperature recordings are 0.5 mm apart in depth. Hp indicates the

location of the hippocampus.

3.1.3   Thermal Imaging

Our video showed a rapid time course of cooling and rewarming. Comparing the images when the loop is off to when it is on shows that the loop has a cooling effect on the surface of the brain tissue (Figure 3.5). The recordings from the time course show that once the cryoloop is turned on for a period of 60 s, tissue will rewarm in a period of approximately 60 s (Figure 3.6). The time course also shows that temperatures dropped below the threshold for cooling (20 °C).
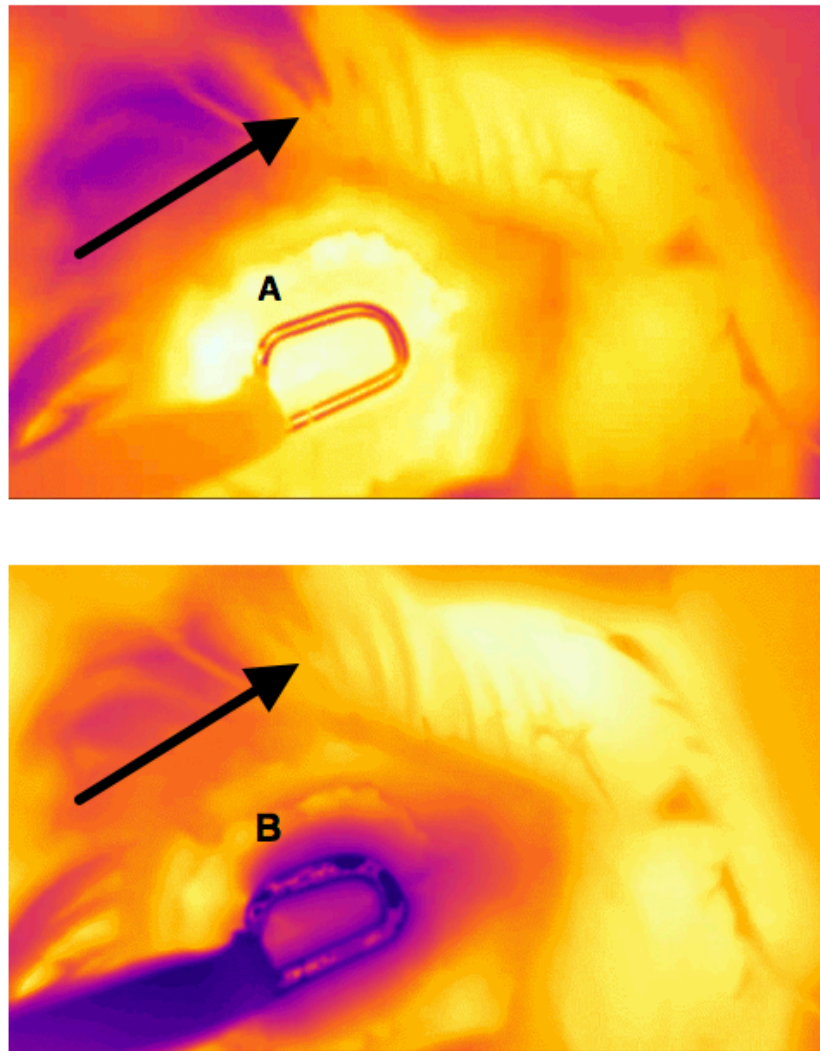
Figure 3.5. Thermal images of a working cryoloop overtop of the brain in a Brown-headed cowbird. Warmer colours denote warmer temperatures. Cooler colours denote cooler temperatures. Arrow denotes the orientation of the head. Top panel: Cryoloop (A) is turned off. Bottom panel: Cryoloop (B) is turned on.

Time Course of Normal Brain Temperature

Time Course of Cooling and Re-warming
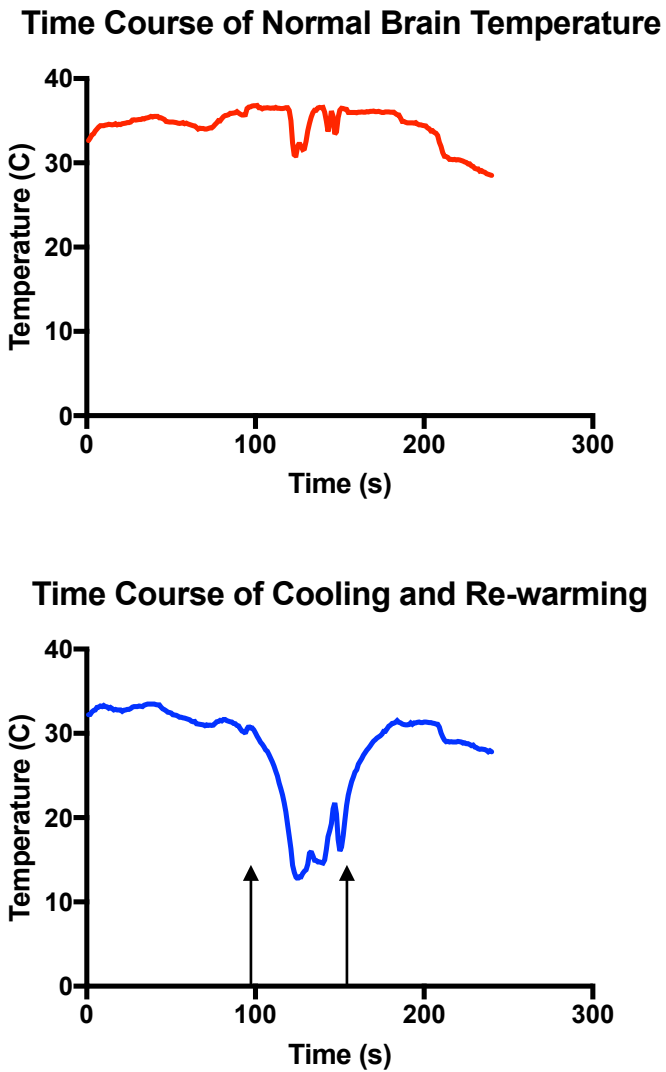
Figure 3.6 Data recorded from thermal image camera. Top panel: time course of Brown-headed cowbird brain while cryoloop was off. Bottom panel: time course of cooling and rewarming of Brown-headed cowbird brain. Space between arrows denotes when the loop was on.

3.2 Tasks

3.2.1 Task acquisition

Brown-headed cowbirds successfully learned both the colour and the spatial touch screen task. Birds were successful in each phase of training. All birds acquired training versions of the task, performing much better than chance levels in each. Birds also performed much better (typically scoring 40% correct each session) than chance (12.5%) on the final (eight choice) versions of the task. I used an online binomial calculator (http://stattrek.com/online-calculator/binomial.aspx) which used total number of trials in a given session, number of trials correct in a given session, and the chance performance on a given trial (12.5%) to calculate the probability of the performance in a single session. If p > 0.01 three sessions in a row, for both tasks individually, it was determined birds had learned the task. This is how my criterion to move on to lesion was determined. In addition to improving on the tasks over sessions, learning the appropriate response within a session was also evident within sessions pre-lesion, as a general learning curve is seen for each bird (see below).

The spatial task was more difficult than the colour task (Figure 3.7). To compare overall performance in sessions across the two tasks I ran a 2 x 5 repeated measures ANOVA (task type x session) using data from the pre-lesion data for the last five sessions prior to lesion for each bird. There was a significant main effect of task $F(1,3)$ =43.16, $p = 0.0072$. No main effect of session number was found $F(4, 12) = 0.7424$, $p=0.5812$, indicating that performance had reached an asymptote by the time of the

lesion. There was not a significant interaction for session x task $F(4,12) = 0.2972$, $p=0.8742$.

Overall Spatial and Colour Task Performance
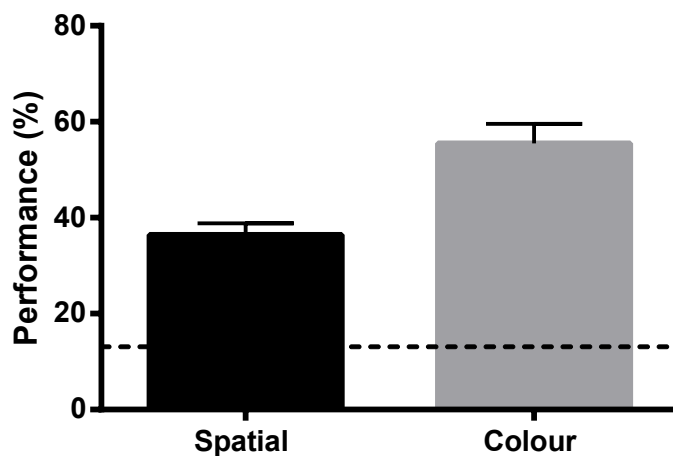
Figure 3.7 Mean performance of all four subjects prior to lesion. Five sessions immediately prior to lesion for each subject are included. Error bars are SEM. Performance was significantly better on the colour task $F(4,12) = 43.16$, $p = 0.0072$. Dotted line is chance (12.5%).s

3.2.2   Effect of lesion

In order to quantify performance I graphed performance throughout sessions for each bird individually. Each bird had its performance averaged across five sessions prior to the lesion, and five sessions post lesion for both the colour and spatial task. The acquisition and retrieval phase (Figures 2.9 & 2.10) were broken into five blocks of four minutes each. The average performance of each bird in each of these blocks of time was calculated across the five sessions pre and post lesion. This allowed for performance

throughout the sessions on the colour and spatial task to be easily compared pre and post lesion. Average trial numbers for each block are also presented for each bird.

To further quantify performance on the phases in a session, I calculated acquisition, retrieval and relearning scores for each subject. All scores are performance percent changes. Acquisition scores were determined by calculating the change in performance between the mean of blocks 1 and 2 and the mean of blocks 4 and 5. Relearning scores were determined by calculating the increase in performance from the averages performance on blocks 6 and 7 to the average performance on blocks 9 and 10. Retrieval was determined by subtracting the performance on block 6 from that on block 5.

In general, three of the four subjects showed no observable detriment in spatial or colour tasks post-lesion. One bird showed observable detriment post lesion in the spatial task, but less so in the colour task. Additionally, this one bird also showed detriments in its spatial acquisition score, but not its colour retention score. Relearning and retrieval were similarly lower post-lesion in the spatial task for this subject. Summaries of the findings from each bird are presented below. Because the sample size is small data are shown for each bird individually.

3.2.2.1   Subject 1

In general, there were no observable difference on the spatial and colour tasks pre and post lesion (Figure 3.8). Performance throughout the spatial and colour task pre and post-lesion are very similar, with no observable difference (Figure 3.8).

Acquisition scores were higher post-lesion than pre-lesion on the colour and spatial tasks (Figure 3.9). Small observable differences show retrieval scores were higher for the spatial task post lesion, with no change colour task post lesion (Figure 3.9). Small observable differences also show relearning was lower for both tasks post lesion (Figure 3.9).
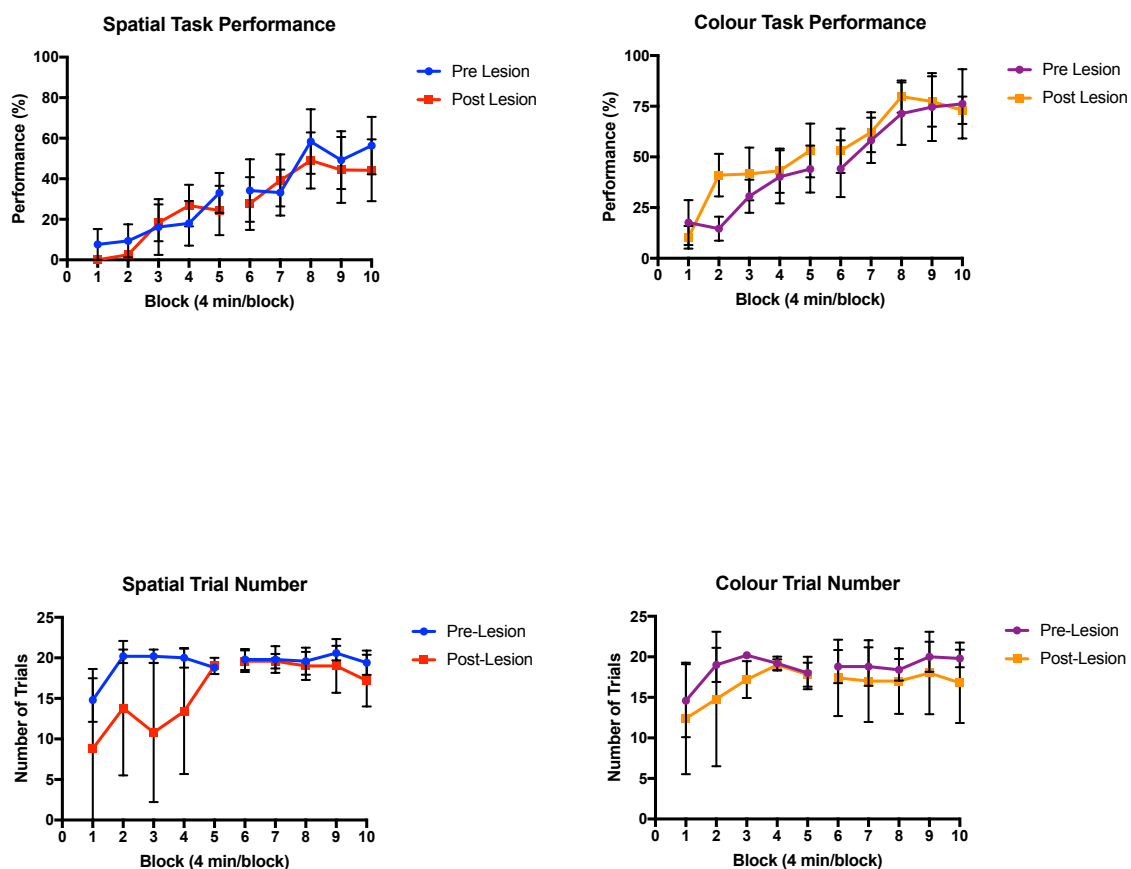
Figure 3.8. Top panels: Bird 1's average spatial and colour task performance across five sessions pre-and post lesion. The first five blocks represent the acquisition phase, the break in the middle represents the retention phase, and the last five blocks represent the retrieval phase. The 20 minute acquisition and retrieval phases were broken into five four minute blocks. Each point represents average performance in each block. Error bars are

SEM. Bottom panels: Bird 1's average spatial and colour task trial number across five sessions pre-and post lesion. Layout is identical to top panels. Each point represents average trial number in each block. Error bars are SEM.

Figure 3.9. Bird 1's acquisition, retrieval and relearning scores for both the spatial and colour task. A higher score indicates better acquisition, retrieval, and relearning. Acquisition scores equal the change in performance between the mean of blocks 1 and 2

and the mean of blocks 4 and 5. Relearning scores were determined by calculating the increase in performance from the averages performance on blocks 6 and 7 to the average performance on blocks 9 and 10. Retrieval was determined by subtracting the performance on block 6 from that on block 5. Performance for each score is the number of correct trials divided by the number of total trials within a session. Error bars are SEM.

3.2.2.2   Subject 2

Performance on the spatial task improved post lesion in the acquisition phase (Figure 3.10). By the end of the retrieval phase in the spatial task, we see similar performance pre and post lesion. Performance on the colour task improved post lesion in the acquisition phase (Figure 3.10). By the retrieval phase, we see similar performance pre and post lesion (Figure 3.10).

Performance observably improved in the acquisition scores post lesion for the spatial and colour task (Figure 3.11). For the spatial task, retrieval was also improved post lesion (Figure 3.11). For the colour task, retrieval was worse post lesion (Figure 3.11). For the spatial task, performance was worse post lesion on the relearning score (Figure 3.11). For the colour task, performance appears slightly worse post lesion on the relearning score (Figure 3.11) due to asymptotic performance (Figure 3.10).

Figure 3.10. Top panels: Bird 2's average spatial and colour task performance across five sessions pre-and post lesion. The first five blocks represent the acquisition phase, the break in the middle represents the retention phase, and the last five blocks represent the retrieval phase. The 20 minute acquisition and retrieval phases were broken into five four minute blocks. Each point represents average performance in each block. Error bars are SEM. Bottom panels: Bird 2's average spatial and colour task trial number across five sessions pre-and post lesion. Layout is identical to top panels. Each point represents average trial number in each block. Error bars are SEM.
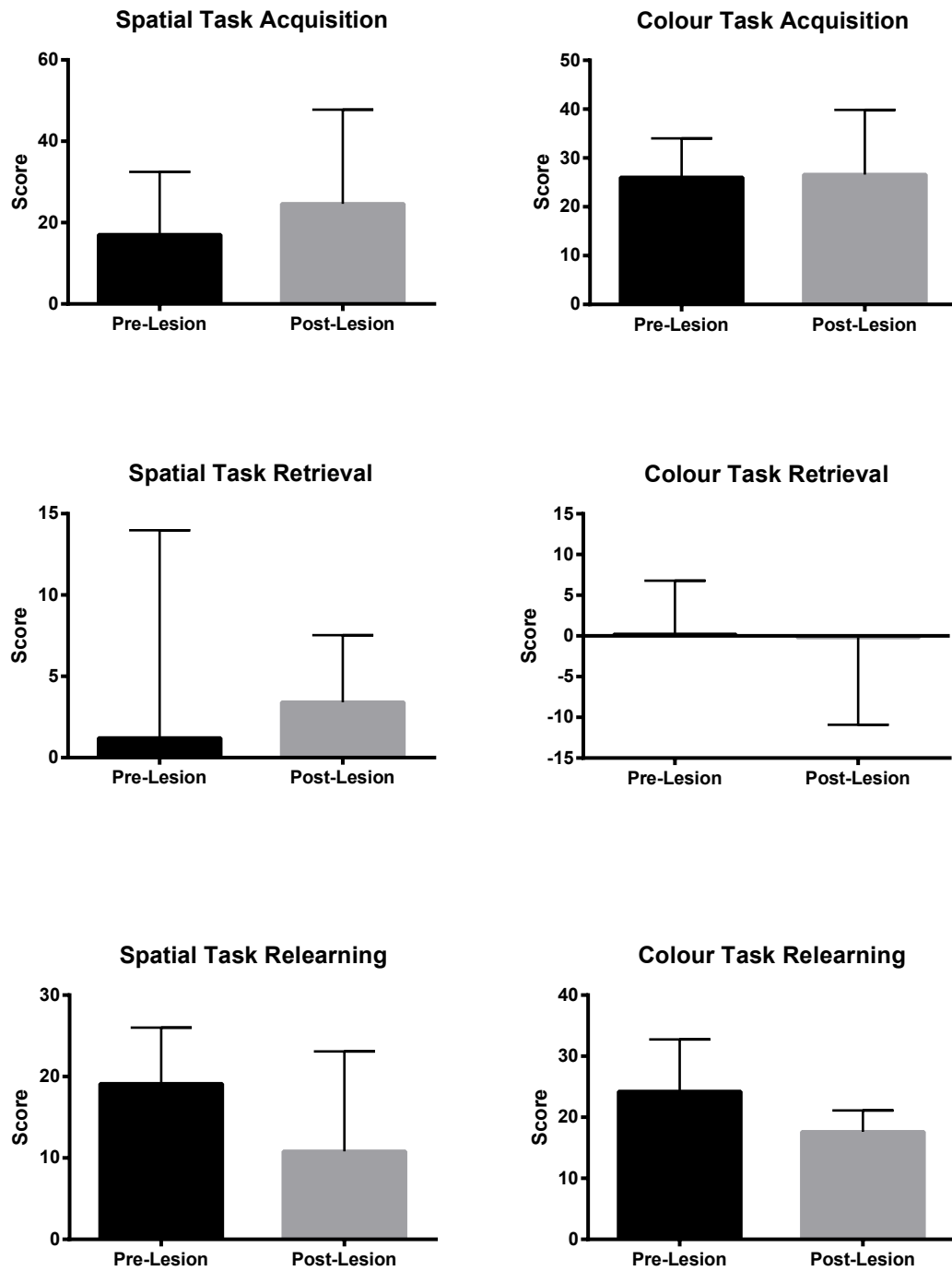
Figure 3.11. Bird 2's acquisition, retrieval and relearning scores for both the spatial and colour task. Score is calculated as described for Figure 3.9. Error bars are SEM.

3.2.2.3   Subject 3

In general, there were no observable difference on the spatial and colour tasks pre and

post lesion (Figure 3.12). Performance throughout the spatial and colour task pre and

post-lesion are very similar, with no observable difference (Figure 3.12).

Acquisition scores were higher post lesion on both tasks (Figure 3.13). Retrieval

in the spatial task improved post lesion (Figure 3.13). Colour task retrieval was worse

post lesion (Figure 3.13). Relearning graphs also showed a similar performance pre and

post lesion on both tasks (Figure 3.13). For the spatial task, there was a slight decrease in

the relearning score (Figure 3.13). For the colour task, there was a slight increase post

lesion in the relearning score (Figure 3.13).
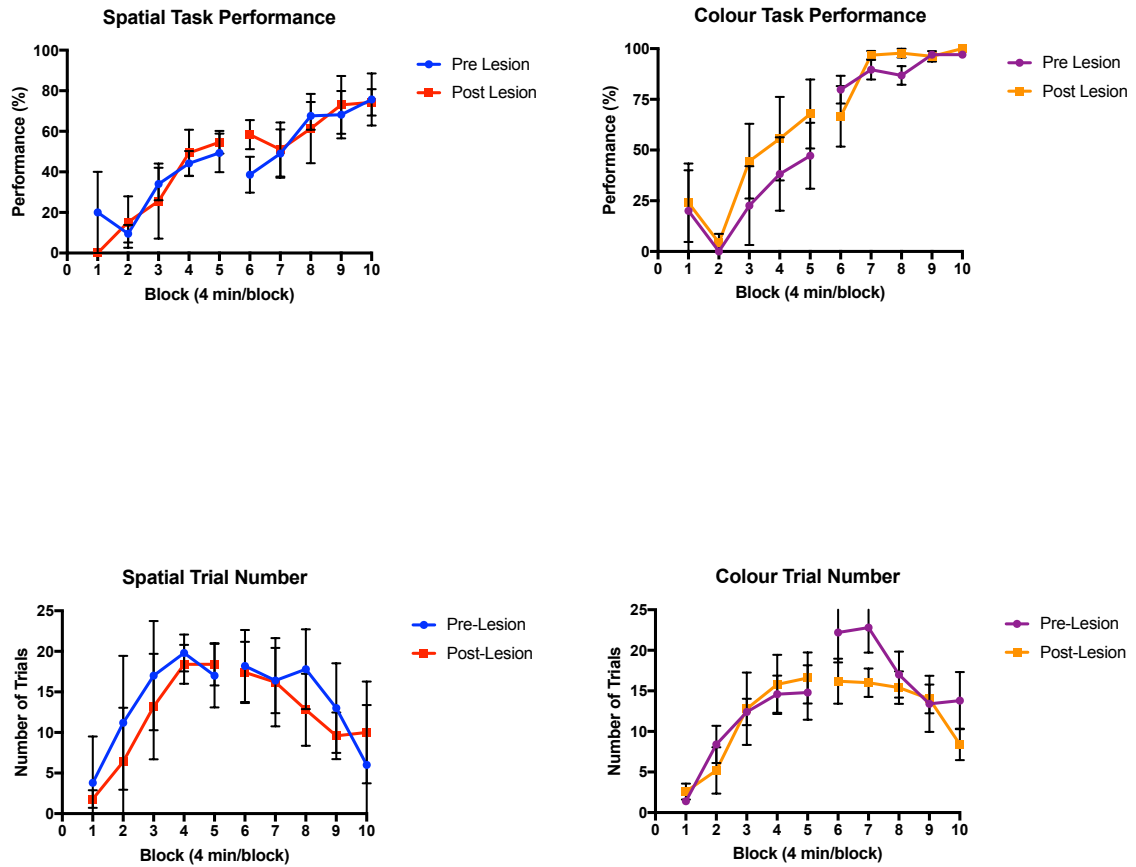
Figure 3.12. Top panels: Bird 3's average spatial and colour task performance across five sessions pre-and post lesion. The first five blocks represent the acquisition phase, the break in the middle represents the retention phase, and the last five blocks represent the retrieval phase. The 20 minute acquisition and retrieval phases were broken into five four minute blocks. Each point represents average performance in each block. Error bars are SEM. Bottom panels: Bird 3's average spatial and colour task trial number across five sessions pre-and post lesion. Layout is identical to top panels. Each point represents average trial number in each block. Error bars are SEM.
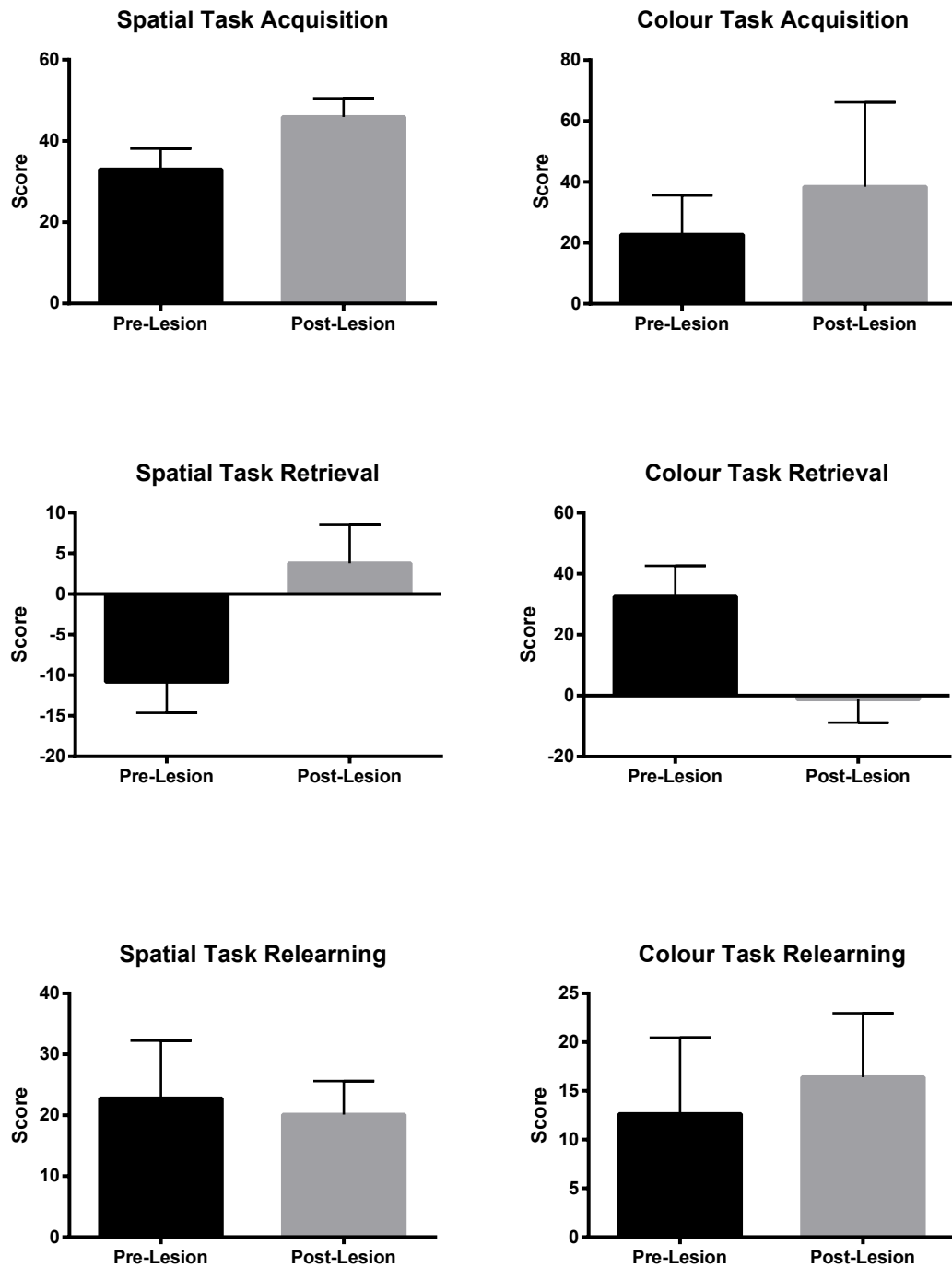
Figure 3.13. Bird 3's acquisition, retrieval and relearning scores for both the spatial and colour task. Score is calculated as described for Figure 3.9. Error bars are SEM.

3.2.2.4   Subject 4

Performance on the spatial task was lower for this subject post-lesion compared to pre-lesion throughout the session (Figure 3.14). Blocks 1-3 show little difference, but by the 5th block the difference becomes clear (Figure 3.14). When the 10th block is compared to the 1st pre-lesion, there is an increase in performance. When the 10th block is compared to the 1st post-lesion, there is little difference in performance. Performance on the colour task was also lower for this subject post lesion compared to pre-lesion (Figure 3.14). Blocks 6-10 show the most differences, whereas blocks 1-5 are more similar (Figure 3.14).

Acquisition scores were lower post lesion for both the spatial and colour task (Figure 3.15). This was the only subject that showed a negative acquisition score in the spatial task (Figure 3.15). Performance was also lower post lesion on the colour task (Figure 3.15). Spatial and colour task retrieval were slightly better post lesion (Figure 3.15). Relearning was lower on the spatial task post lesion (Figure 3.15). Relearning was similar on the colour task post lesion and pre lesion (Figure 3.15).
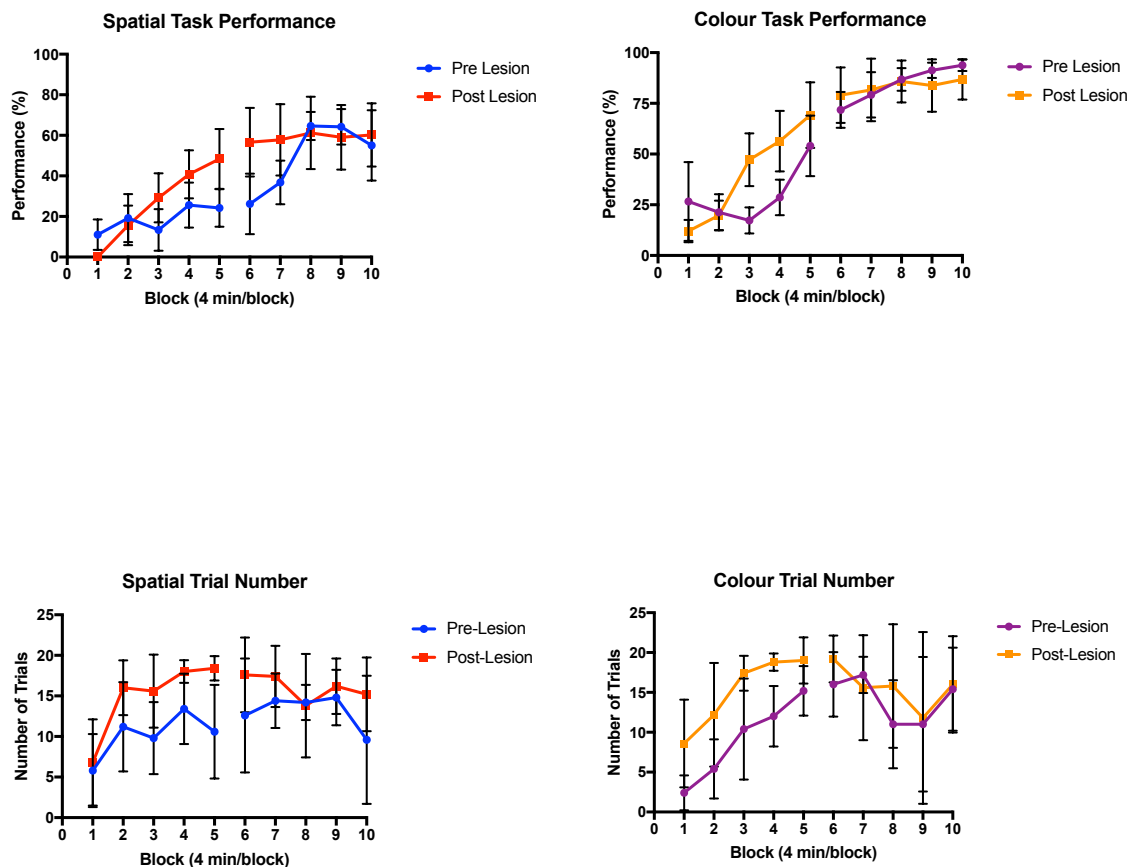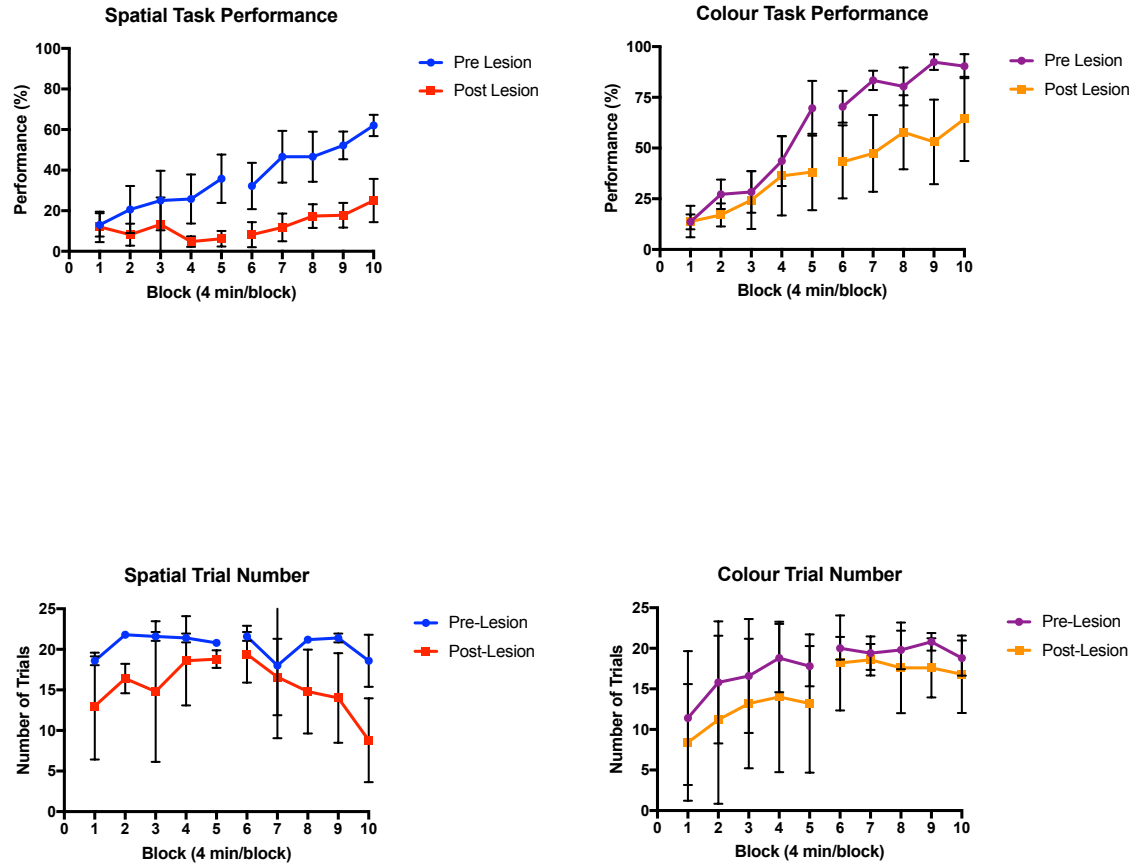
Figure 3.14. Top panels: Bird 4's average spatial and colour task performance across five sessions pre-and post lesion. The first five blocks represent the acquisition phase, the break in the middle represents the retention phase, and the last five blocks represent the retrieval phase. The 20 minute acquisition and retrieval phases were broken into five four minute blocks. Each point represents average performance in each block. Error bars are SEM. Bottom panels: Bird 4's average spatial and colour task trial number across five sessions pre-and post lesion. Layout is identical to top panels. Each point represents average trial number in each block. Error bars are SEM.
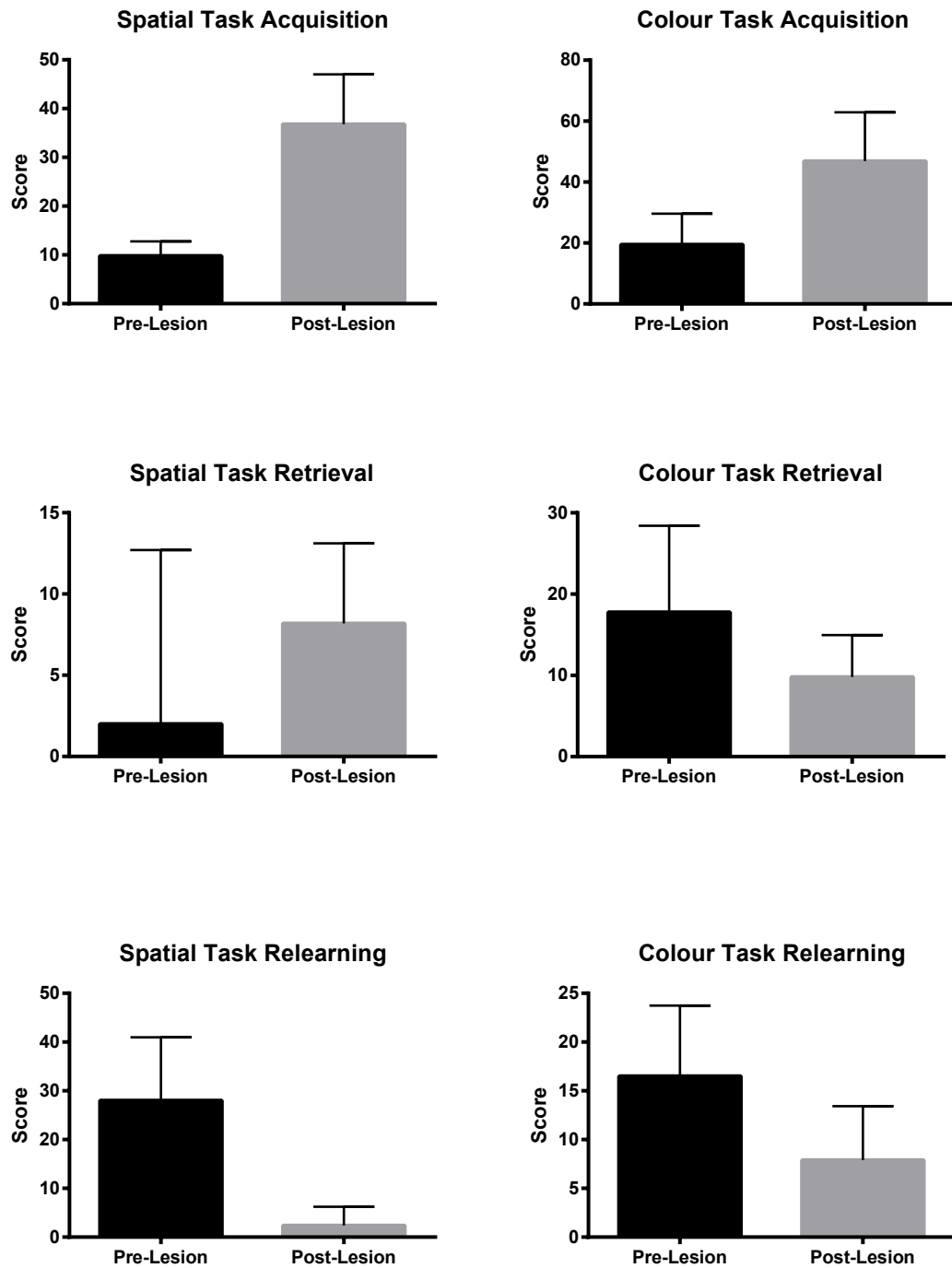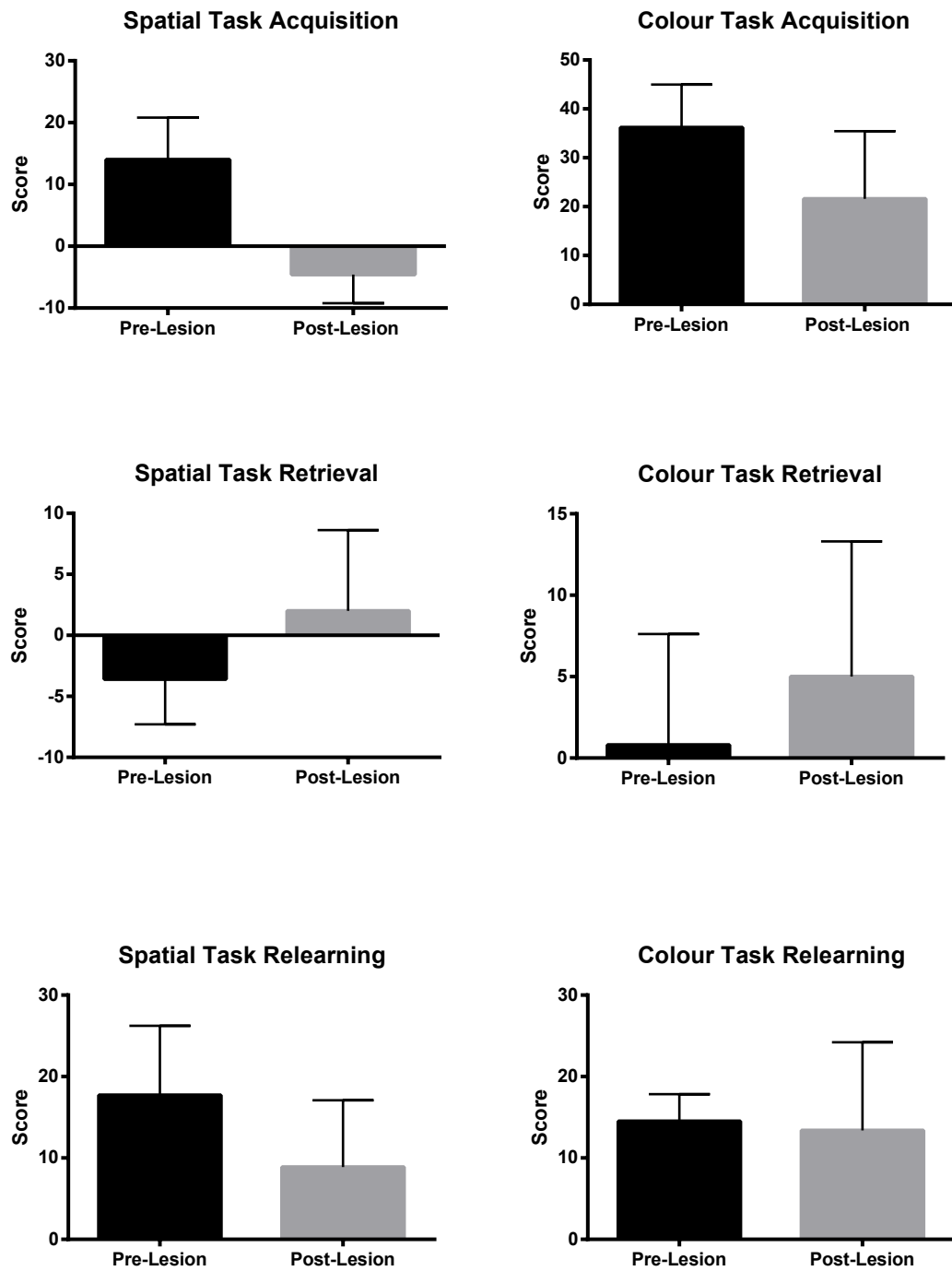
Figure 3.15. Bird 4's acquisition, retrieval and relearning scores for both the spatial and colour task. Score is calculated as described for Figure 3.9. Error bars are SEM.

3.2.3   Lesion verification

Ibotenic acid lesions were not visible in Nissl-stained tissue in any of the four subjects,

potentially as a result of the brains being collected 16-26 days after lesion. At the time of

brain collection, the holes in the skull were visible but fully healed and appeared to be

located directly above the hippocampus. Injection tracks from the lesion syringe were

visible in each bird. All tracks were not visible, but at least two tracks were found in each

subject (Figures 3.16 & 3.17). Tracks were estimated to be in the correct locations

anterior to the sinus (1.5 & 3.5 mm anterior), with most error being that the needle tracts

were placed farther laterally from the midline than intended (Figures 3.16 & 3.17).

Mechanical damage was found in Bird 4 (Figures 3.16 and 3.18).
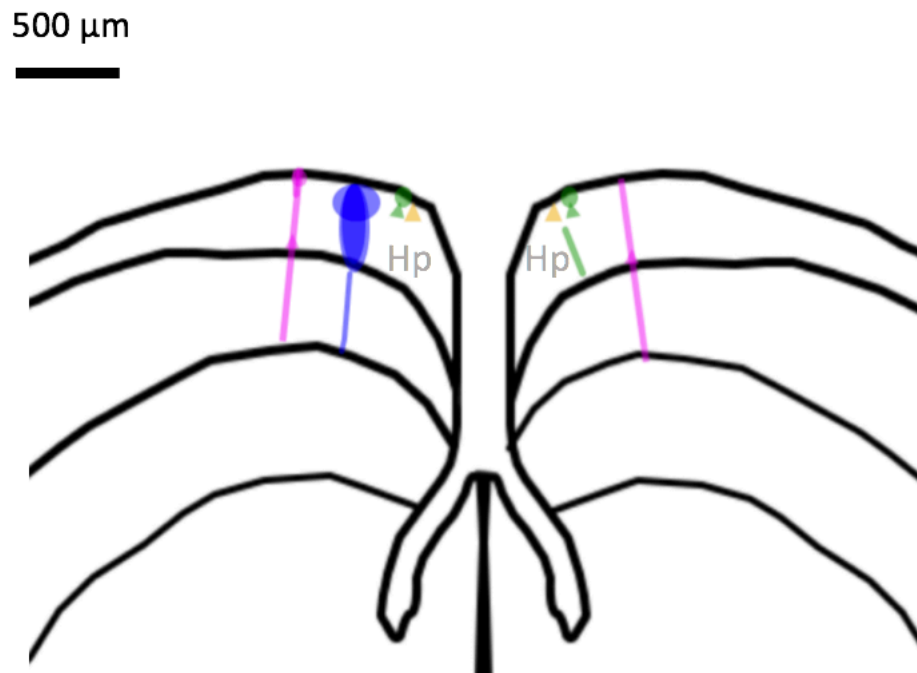
Figure 3.16. Track and damage locations overlaid on Brown-headed cowbird brain tracing (coronal; 1.5mm anterior to sinus). Different colours represent different subjects. Pink denotes Bird 1, green denotes Bird 2, yellow denotes Bird 3, and Blue denotes Bird 4. Hp indicates hippocampus.

2000 μm
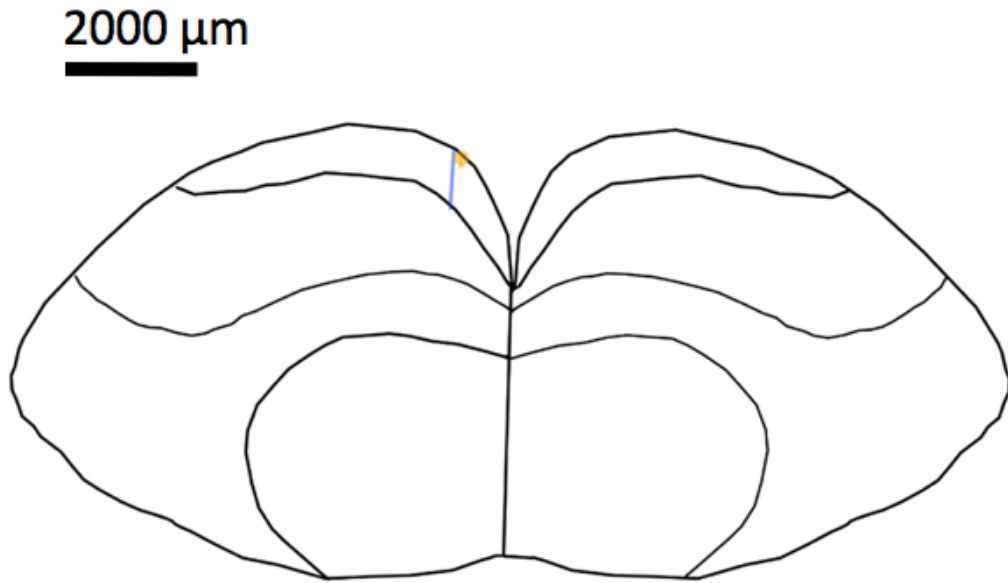
Figure 3.17. Track and damage locations overlaid on Brown-headed cowbird brain tracing (coronal; 1.5mm anterior to sinus). Different colours represent different subjects. Yellow denotes Bird 3, blue denotes Bird 4.
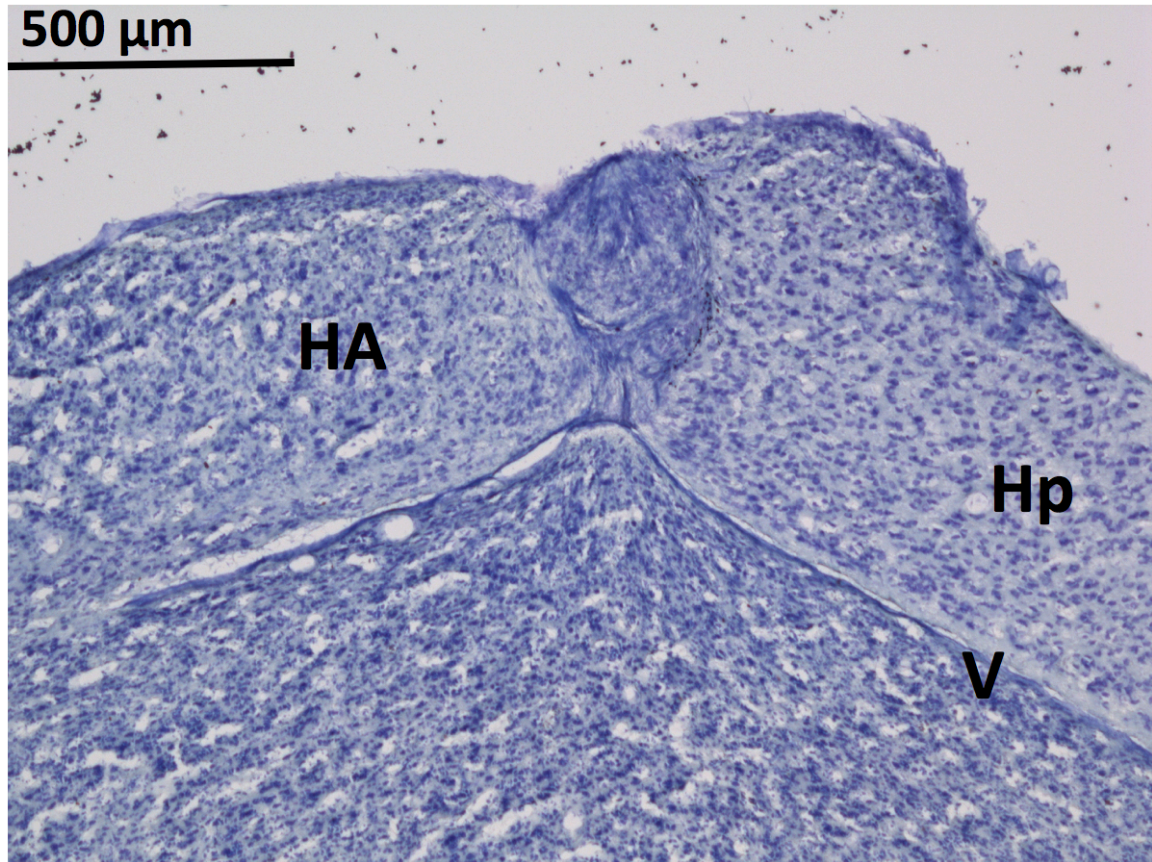
Figure 3.18. Photomicrograph of observed mechanical damage from needle during lesion procedure in Bird 4 (coronal;1.5 mm anterior to sinus). Tissue stained for Nissl. Thickness = 40 µm. Hp indicates the hippocampus, V indicates the ventricle, Ha indicates Hyperstriatum Accessorium.

Chapter 4

4   Discussion

4.1   Cryoloops

While performing cryoloop installation surgeries, we encountered issues with how to fix the cryoloop to the skull. Cryoloops have only been fixed to mammals, and the makeup of the mammalian skull is different than the avian skull. In birds, the skull is pneumatized (Simonetta, 1960). This makes for a brittle structure to work with, and thus is difficult to anchor. Due to this pneumatization, a screw cannot be drilled to anchor the cryoloop. We determined that the best method of fixing the cryoloop to the skull was by threading small wires through drilled holes on the outside of the craniotomy. Wires on one side stood straight up, while on the other side, the wires bent over the cryoloop. In this way, the wires acted like rebar in concrete. Dental cement was placed over the skull, the wires, and the cryoloop, and we were able to fix the cryoloop strongly to the skull. Others have attached a variety of different neural devices to bird's skulls, such as deep electrodes and microdrives. Many procedures only use dental cement (Roberts, Gobes, Murugan Ölveczky & Mooney, 2012) and an epoxy (Kao, Wright, & Doupe, 2008), with no additional anchor, while others do use some additional type of anchoring to fix their devices to a bird's skull (Ter Maat, Trost, Sagunsky, Seltman & Gahr, 2014; Guitchounts, Markowitz, Liberti & Gardner, 2013). Considering there have been successful attachments in bird skulls, our method should be possible.

Following several pilot non-recovery surgeries, I successfully had one animal

recover from surgery with the cryoloop fixed in this way. This subject was the only one where the attachment did not immediately detach when pressure was applied to it following surgical attachment. Although initially successful, this attachment failed approximately 3 days after attachment and the bird was immediately euthanized. It is likely that the cryoloop detachment occurred due to the size of the implant (Figures 2.2 and 3.1). In particular, the tube and temperature connecters extended approximately 2 cm (Figure 2.2). These long connectors would create substantial leverage if the bird applied force to the end of the structure. Therefore, our next step is to make the attachment more compact. The length of the attachment was such that it was likely easy for the subject to remove the attachment. Combining our anchoring method with a smaller attachment could produce better results.

I determined that cryoloop cooling cools brain tissue in the Brown-headed cowbird. The closer a temperature probe was to the cryoloop, the cooler the tissue, in both depth and lateral distance. The tissue was also able to rewarm to its regular temperature within approximately one minute.

We found that in our first temperature probe recordings, as well as with our thermal imagining video, that the cryoloop's arms were too wide to properly target the hippocampus. Our solution was to place one arm of the cryoloop above the midline of the brain. Additionally, I noticed one of the greatest challenges in surgery was removing the inner layer of skull. Removing the inner layer of skull was by far the longest part of the procedure, and was difficult to remove completely. Taking both of these points into consideration, I decided to try the new orientation of the cryoloop directly above the inner layer of the skull. During the temperature probe recordings with the new

orientation, the bird died while temperature probes were taken, and I was not able to have periods of rewarming between each temperature recording. I still decided to take multiple recordings at different depths of the brain. I found the cooling to be directed to hippocampus; the cooling did not extend to a depth past 1 mm at our recording of 0.5 mm lateral to the midline. This new placement of the cryoloop will allow for more accurate cooling. Additionally, placing the cryoloop above the inner layer of skull allows for protection of any damage to the brain tissue. Therefore, animals should live with the probes implanted longer, and can be used in experiments in the long term.

Next steps for the cryoloops are to implant new smaller cryoloop into more Brown-headed cowbirds, and have a subject cooled while awake. Next, I will observe how a bird behaves in its home cage while being tethered to the apparatus. Lastly, I will observe how a bird behaves in its home cage while having the hippocampus cooled.

There are many scientific advantages that come with successful cryoloop implantations in birds. The hippocampus as a structure can be investigated in new ways, and we will be able to answer new and interesting questions about the hippocampus. With cryoloops, birds can be used longer term in experiments. This method can also be extended for cryoloops in different avian species. This method can also be extended for cryoloops to cool different regions in the avian brain, such as HVC, a structure important in the song control system.

4.2   Touchscreen tasks

Birds' performance on the colour task was significantly better than bird's performance on the spatial task. A modification of the tasks to make the spatial and colour tasks more comparable on performance is necessary for the future. This would allow for the colour task to be a better control to the spatial task.

Considering Bird 4 had damage in the hippocampus, and a decrement in spatial task performance post lesion, further pursuing the validity of this task is necessary. While no ibotenic acid lesions were visible, a large amount of mechanical damage was found in Bird 4. This was the largest amount of damage I found across all birds. This damage was not in the darkly staining V section of the hippocampus, and only found on the left side of the brain. This bird was also the only subject which showed considerable reduced performance post-lesion. If the task is hippocampus dependent, as this result implies, then future lesions with successful ibotenic acid should produce much more pronounced detriments on the spatial task. I could not find any damage in the three other subjects, only needle tracks. These birds did not show a decrement in performance post-lesion. These results also match with the prediction that the spatial task is hippocampus dependent. A lack of decrement post-lesion in these three subjects also demonstrates that surgery itself does not have an impact on performance on the tasks. If this task is hippocampus dependent, it will be useful as hippocampus dependent egocentric tasks are less common than hippocampus dependent allocentric tasks (e.g. Colombo, Cawley & Broadbent, 1997; Colombo & Broadbent, 2000; Mayer *et al*., 2012; Watanabe, 1999).

I was not able to successfully identify any ibotenic lesions in any of my birds. It is possible that this was because birds were sacrificed 16-26 days post-lesion. I noticed that in all subjects, the holes of the skull had healed. This longer period of time could be an explanation as to why it was difficult for me to locate any lesions, and many of the needle tracks. Perhaps over this time, enough neurogenesis had occurred to hide the damage caused by lesions and needle tracks. While there is not a known time estimate for the migration of new neurons to the avian hippocampus, estimates for migration of new neurons to the HVC is around 9-15 days (Kirn, Fishman, Sasportas, Alvarez-Bullya & Nottenbohm, 1999). The needle tracks that were located do provide a good indication of where the lesions were directed. Our coordinates for the lesions were 0.5 mm lateral to the midline, 1.5 mm & 3.5mm anterior of the central sinus, bilaterally, for a total of four holes. The location of the needle tracks anterior to the sinus were estimated to be accurate to our coordinates. However, we were more consistently too lateral to the midline, our farthest track being 1.5 mm lateral to the midline (Figure 3.16). I am reviewing my lesion procedure to hit the coordinate accurately, by injecting acid on the edges of our drilled holes, which themselves are about 0.5-1 mm in size.

4.3   Cryoloops in conjunction with the touchscreen task

The future of the cryoloops and the touchscreen tasks both look promising from what has been presented here. It is possible to cool the tissue, and it seems hopeful that the task may be hippocampus dependent (based on Bird 4 results). The progress made with the cryoloops and touch screen task brings us closer to answering the research question:

"What is the role of the hippocampus in encoding, retention and retrieval of spatial information in Brown-headed cowbirds?".

If we assume that the task is hippocampus dependent, next we will observe if birds can simply work on a touchscreen and acquire a food reward with the probe attached. The following step will be to observe if birds can perform the final versions of the tasks while tethered to the outside apparatus. Next, the final goal can be achieved. Birds with successfully implanted cryoloops will perform the tasks and will have their hippocampus cooled and rewarmed during different phases of the task. During testing, the task will not change, however, birds will undergo periods of cooling and rewarming during these three different stages of the task, and their performance on the task will be measured. In total there will be eight different possible combinations of cooling and no cooling during encoding, retention, and retrieval (Figure 4.1). In this way, the experiment is completely within-subjects, with the birds' own un-cooled hippocampus acting as the control condition. Cooling will occur in any of these 8 combinations over a period of a few weeks, as to have periods of no cooling in between cooling days, while still being attached to the apparatus.

In the future, I can also alter the length of different phases of the task, such as using a longer retention phase, similar to Shiflett *et al.* (2003) to investigate long-term vs short term memories. In this case, perhaps birds would perform well on the task even when the hippocampus is cooled during the retrieval phase. An open field task could also be used, to see if there are differences in the involvement of hippocampus in each of these phases for an allocentric task.
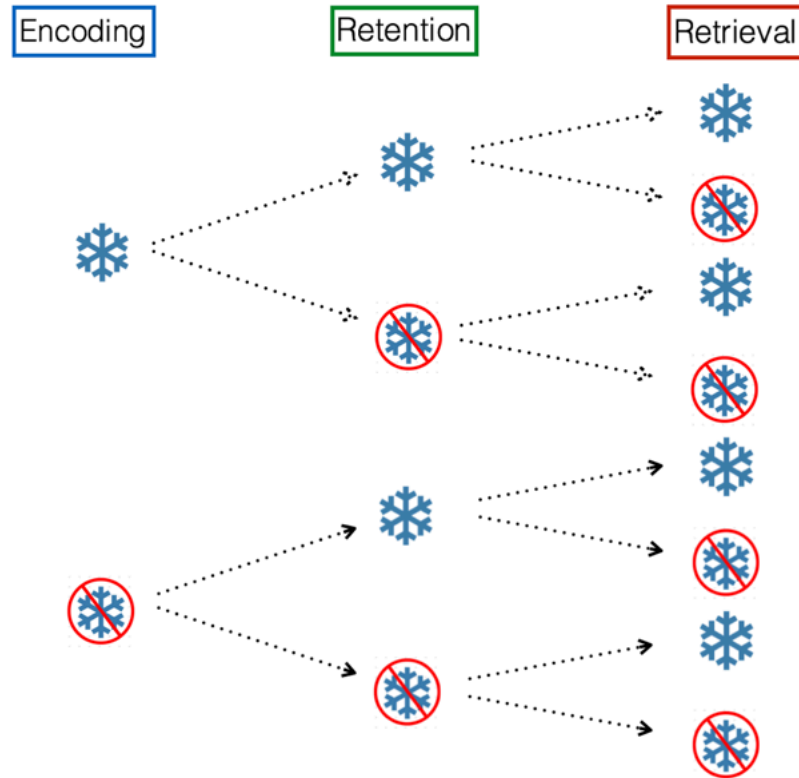
Figure 4.1. Proposed experimental design for future study. Snowflakes denote cooling during a phase, snowflakes with an x denote no cooling. Arrows denote progression through phases.

4.4   Concluding statement

I have laid the groundwork for the proper implantation of cryoloops, as well as a hippocampal dependent task that can be used to answer a question about hippocampal spatial memory formation. The opportunities of studying hippocampus with the cryoloops are vast, as well as the opportunities with a hippocampus dependent touchscreen task in Brown-headed cowbirds.

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Appendix

Western

AUP Number: 2015-088 **PI Name:** Macdougallshackleton, Scott A

AUP Title: Hippocampus Function In Birds **Approval Date:**
03/03/2016

Official Notice of Animal Use Subcommittee (AUS) Approval:
Your new Animal Use Protocol (AUP) entitled "Hippocampus Function
In Birds" has been APPROVED by the Animal Use Subcommittee of the
University Council on Animal Care. This approval, although valid for
four years, and is subject to annual Protocol Renewal.2015-088::1

1. This AUP number must be indicated when ordering animals for this
project. 2. Animals for other projects may not be ordered under this
AUP number. 3. Purchases of animals other than through this system
must be cleared through the ACVS office. Health certificates will be
required.

The holder of this Animal Use Protocol is responsible to ensure that all
associated safety components (biosafety, radiation safety, general
laboratory safety) comply with institutional safety standards and have
received all necessary approvals. Please consult directly with your
institutional safety officers.

Submitted by: Copeman, Laura on behalf of the Animal Use
Subcommittee University Council on Animal Care

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
PH: 519-661-2111 ext. 86768 ● FL 519-661-2028
Email: auspc@uwo.ca ● http://www.uwo.ca/animal/website/

Curriculum Vitae

Madeleine Isabelle Robin Brodbeck

Personal:

Date of Birth: September 1st, 1993

Birthplace: London, Ontario

Citizenship: Canadian

Languages: English, French

Education:

Master of Science, Behavioural and Cognitive Neuroscience, Psychology, Western University, Expected 2017.

Bachelor of Science, Honours, Psychology, 2015, Algoma University.

Research Experience

Dates	Institution	Supervisor	Description
September 2015 – Present	Western University	Dr. David Sherry & Dr. Scott MacDougall-Shackleton	Master's thesis on spatial memory in brown-headed cowbirds
September 2014 – May 2015	Algoma University	Dr. Paul Dupuis	Undergraduate Honour's Thesis on video games and visual attention
May 2014 – August 2015	Algoma University	Dr. Laurie Bloomfield	Research on chickadee communication

| June 2013 – August 2013 | Algoma University | Dr. Dwayne Keough | Research on motor control in golfing |
| May 2012 – August 2012 | Algoma University | Dr. Dwayne Keough | Research on motor control in singers |

Teaching Experience

Dates	Institution	Position	Responsibilities
April 3rd 2017	Brescia University College	Guest Lecture	Guest lecture for psychology research methods
January 2017 – April 2017	Western University	Teaching Assistant	Grading, exam proctoring, meeting students
January 2016 – April 2016	Western University	Teaching Assistant	Grading, exam proctoring, meeting students
September 2014 – April 2015	Algoma University	Head Psychology Teaching Assistant	Grading, exam proctoring, meeting students, running tutorials, organizing & mentoring other TAs

September 2013 – June 2014	Algoma University	Tutor	Tutoring for students in Intro Psychology
September 2012 - April 2014	Algoma University	Teaching Assistant	Grading, data entry, meeting students

Scholarships & Awards

Name of Award	Institution	Monetary Value/year	Dates
Ontario Graduate Scholarship	Western University	$15000	September 2016- August 2017
NSERC Canada Graduate Scholarship - Master's	Western University	$17500	September 2015 - August 2016
NSERC Canada Graduate Scholarship – Master's (**not accepted**)	Dalhousie University	$17500	Not accepted
Award of Excellence in Psychology (science honours)	Algoma University		June 2015
The Governor General's Academic Medal (Silver)	Algoma University		June 2015

NSERC Undergraduate Student Research Award	Algoma University	$4 500	May 2015 – August 2015
NSERC Undergraduate Student Research Award	Algoma University	$4 500	May 2014 – August 2014
Edward and Frank McGrath Award of Excellence	Algoma University	$3 750	September 2011 – May 2015

Publications

Brodbeck, MIR and Brodbeck, DR. (2015). It's just evolution. Commentary: A crisis in comparative psychology: where have all the undergraduates gone? *Front. Psychol.* 6:1873. doi: 10.3389/fpsyg.2015.01873

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