Life without cortex:
Subcortical circuits in naturalistic behaviors

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ABSTRACT

A major goal of neuroscience is to understand the neural circuits underlying animal behavior. Many contemporary studies focus on behavioral tasks which do not reflect realistic conditions, such as mapping an arbitrary sensory stimulus to motor output. Given that the brain evolved within the context of the natural environment, it is more likely that these circuits were optimized for naturalistic behaviors such as avoiding predators, hunting, and social interactions with conspecifics. Many of these naturalistic behaviors predate the great expansion of the neocortex in mammals, as they are crucial for the survival of any animal. Using a mutant mouse model and surgical techniques, we show that the evolutionarily ancient subcortical circuits of mice are sufficient for sensory processing, stimulus discrimination, and exhibiting robust innate defensive behaviors in a predator avoidance assay. Furthermore, these animals are capable of navigating a complex labyrinth, which challenges long-held beliefs that learning and memory require the neocortex and the hippocampus. Our results emphasize the significant capacity of subcortical circuits in behaviors necessary for survival and illustrate the importance of using naturalistic behaviors to probe brain function.

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Chapter 1

INTRODUCTION

Evolution favors traits that ensure survival. Despite its energy expenditure and maintenance cost, the great expansion of the neocortex in the past 220 million years must be indispensable to mammals [20]. The emphasis on the investigation of cortical circuits in neuroscience research is aligned with the idea that these recent inventions of evolution are vital. This comes as no surprise given that these are the regions that went through a rapid evolutionary process which led to the species of the researchers. For more than a century, understanding the function of the cortex has been a major focus in experimental research [21, 38]. With the advancement of multielectrode probes [31, 63], improved calcium indicators [11], and new imaging techniques [32], many contemporary research questions rely on recording from a given cortical area as the animal is being presented a specific stimulus with the aim of understanding the computation underlying the stimulus-dependent response [25, 76]. In addition to being the most recent invention of evolution, the neocortical and hippocampal structures are attractive regions to study due to their localization within the mammalian central nervous system providing easy access for functional manipulations and recordings.

Given that the ancestral organisms must have had the capacity to survive prior to the emergence of these relatively new circuits, how much of an animal's behavior is dependent on the neocortex and hippocampus? Before we try to answer this question, it is important to state definitions clearly. The interpretation of behavior is a heavily debated subject in neuroscience. We focus on naturally occurring behaviors including predator avoidance [74], foraging and hunting [28], nesting [12], burrowing [19], sleeping [65], parental behavior [67], and social interactions such as aggression and courtship behaviors towards conspecifics [33, 44]. Some of these innate behaviors require some learning whereas other circuits can be functioning without any prior experience. Some of these tasks such as sleep, foraging, and nesting can be performed under relatively more flexible conditions whereas some require immediate action to ensure well-being and survival. One could argue that among all of the natural behaviors that a mouse has to perform successfully to survive, predator avoidance is the most immediate and crucial. This is true for all animals: Avoiding predators is a task with zero tolerance for failure.

In the laboratory setting, predator avoidance has been studied in many species including fruit flies [8] and zebrafish [15]. In laboratory conditions, these animals are exposed to an expanding dark disk which mimics an approaching predator. This looming stimulus triggers robust escape behaviors which are thought to be regulated by innate defensive circuits. Previously, it has been shown that wildtype mice respond to this stimulus in laboratory conditions as well [74] by either freezing or escaping to shelter. This universally conserved animal behavior is an ideal candidate paradigm to ask the original question with two complementary parts: *What is the capacity of subcortical circuits in predator avoidance behaviors of the mouse? What is the role of newly evolved mammalian brain structures in this crucial behavior*? To test this, we would need to eliminate all neocortical and hippocampal circuits.

Removing all neocortical and hippocampal tissue from a wildtype mouse is a technical challenge. Reversible methods for inactivating these circuits would require the effector agent to be expressed or the inhibitory drug to be infused throughout the neocortex and hippocampus. Then, the extent of inactivation would have to be validated within every substructure. Given the required extent of these manipulations, we decided to use structural mutants which fail to develop their neocortex and hippocampus [34]. We challenged the acortical mutants and wildype mice in the looming assay. We have found that these mutants can appropriately detect visual stimuli, discriminate between behaviorally relevant and irrelevant visual stimuli, and execute appropriate motor outputs based on the sensory input. The acortical mice, however, show deficits in directed navigation to a shelter when exposed to the looming stimulus and are resistant to adaptation in response to multiple exposures within the same trial. Various positive reinforcers to lure the mutant mice to the shelter for safety did not have an effect suggesting a malfunctioning spatial learning and goal-directed navigation. Surprisingly, when the acortical animals were challenged with exploring a complex labyrinth and locating a reward, all animals were able to learn the reward location and navigate the labyrinth albeit with long delays. Acortical mice exhibited local exploration of the labyrinth without branching out to other sections resulting in the observed delay in finding the reward location. In a new environment, one can define a measure for efficiency of exploration indicative of the new end nodes discovered in a labyrinth given the end nodes that were visited. This measure of efficiency was previously reported for the wildtype animals [60]. The report indicated that the wildtype animals showed a slight decrease in their efficiency of exploration throughout the experiment which lasted overnight. Acortical mice exhibited low efficiency of exploration of the labyrinth during initial phases of

the experiment. However, later in the experiment, acortical mice had the same level of efficiency exploring the labyrinth as the wildtype animals did.

These results suggest that a cortex-independent visual processing and escape circuits are intact within the remaining subcortical regions. Although navigation to shelter is impaired in these animals when facing an immediate threat, the acortical mice are capable of exploration and goal-directed navigation, indicative of capacity for learning independent of the neocortex and hippocampus. The goal of this thesis work is to simulate naturalistic events that an animal faces in its lifetime under laboratory conditions. Using surgical and genetic methods, I studied the capacity of subcortical circuits necessary for survival by testing mice without neocortex and hippocampus by using acortical mice.

1.1 Naturalistic Behaviors Necessary for Survival

The typical wildtype mouse in the laboratory setting does not have to face the natural environment, and in that sense it is far from a wild mouse. However, in the laboratory we are able to simulate certain conditions that mimic what the animal would have experienced out in nature. The natural predators of mice include foxes, snakes, and birds of prey. When avoiding terrestrial predators, mice can detect odors produced by the cats, foxes, snakes and can avoid these environments [58]. Mice are also sensitive to auditory cues such as rat vocalizations and exhibit defensive behaviors when exposed to these vocalizations in the laboratory setting [53]. All aerial attacks arrive from the upper visual field of the mouse and the main sensory modality of this type of attack is vision in these cases. The moment of attack can be simulated by showing a dark expanding disk on a monitor above the animal mimicking an approaching aerial predator.

Organisms survived throughout evolution under varying circumstances leading to the diverse organisms that we currently study. The attributes that were kept around were the ones that gave an advantage for survival. Avoiding becoming food is as important as finding food. In terms of foraging, the animals will have to sample and find sources of energy. The feeding of mice includes small insects. In the laboratory setting, although the test subjects never experienced a need or circumstances which required foraging and hunting, we can motivate the mice by restricting their food intake.

Animals must constantly utilize their guidance systems to navigate efficiently under different circumstances, whether it is to forage for food or to locate their nest or

to escape to safety when under attack. Although spatial learning has been mostly studied under limited experimental conditions such as navigation in open-field box and in linear tracks, more recent advances in virtual mazes [46] and complex labyrinths [60] will provide us better understanding of circuits involved in navigation and goal-learning.

Here, we study normal and mutant animals with missing cortical tissue to answer the question: *what are the advantages of having a cortex in nature?* We simulate naturalistic scenarios in the laboratory setting and distinguish the similarities and differences between the two types of animals: wildtype and acortical.

1.2 Mammalian Subcortical Structures Implicated in Naturalistic Behaviors

Many scientific reports aim to elucidate a causal relationship between stimulus input and behavioral outcome. A large body of work has been done to investigate different subcortical circuits. Hypothalamic circuits have been shown to regulate multiple naturalistic behaviors tested in the laboratory setting. Agrp+ neurons in arcuate nucleus regulate hunger [36]. Within the subfornical organ of the hypothalamus, two groups of neurons were identified: one that promotes drinking behavior and the other suppresses it [56]. The ventromedial hypothalamus (VMH) has been shown to regulate social interactions with conspecifics. A group of neurons expressing Esr1 gene within the ventrolateral part of VMH can trigger attack behaviors [44]. Hunting-like actions are thought to be mediated via a subset of neurons in the medial preoptic area (MPA) which project to periaqueductal gray (PAG) [59]. More recently, different cell types in superior colliculus have been shown to contribute to the hunting behavior [29]. Similarly, predator avoidance behavior has been extensively studied within the superior colliculus and PAG via circuit manipulation techniques [62, 72], functional imaging in freely moving mice [17], and extracellular recording techniques in head fixed mice [42].

Given the overwhelming significance of subcortical regions implicated in the behaviors necessary for survival, we decided to investigate the aspects of behavior that can be accomplished with only the subcortical circuits in a mouse. This is not to say that the cortical and hippocampal circuits are not necessary or inactive during these naturalistic behaviors in wildtype mice. This project is aiming to look for neural correlates of different aspects of behavior that can be performed by the subcortical circuits. By understanding the capacity of subcortical circuits first, we can then make unbiased inferences on the contribution of cortex in different animal models.

It should be noted that during my thesis, I have encountered different schools of thought trying to define what a behavior is and how it should be investigated. The approach taken in my studies has been more on the observational side, surveying different behaviors in an animal model that would be very difficult to generate using any other method.

1.3 Cortical Significance in Rodent Studies

Research suggests that cognition, learning, and decision-making are governed by cortical circuits. These are usually based on studies that can require tens of thousands of trials which last for many weeks [25, 76]. Despite extensive training, the animals' performance remains below what would be considered sufficient if the task was crucial for survival. Arguably, the likely explanation for this low performance is the artificial experimental setup. The animal is expected to learn to associate a sequence of sensory events that carry relevant information and report one out of two options to collect a reward. Experimental paradigms like these do not reflect any behaviorally relevant conditions that the animal could encounter in nature. Moreover, one cannot rule out the possibility that the strong correlations observed in a cortical circuit are due to the extensive training of a specific task [14] and that the observed activity of these circuits may not reflect their function under naturalistic conditions.

The increased interest in studying cortical circuits can be easily observed by quantifying the publications based on cortical circuits over time (Figure 1.1). Inspired by humans, prefrontal cortex has specifically attracted many rodent neuroscientists since the 1990s [41]. With the advancement of available molecular and genetic tools and the convenience of breeding and handling, mice will soon surpass other model organism such as rats and non-human primates in terms of their experimental status in understanding the function of prefrontal cortex in mammalian nervous system.

We should note that within the field of neuroscience, there is ambiguity of cortical regions, especially the prefrontal cortex which is commonly implicated in executive function and decision-making. Research has shown that depending on which rodent atlas is used ([Paxinos and Watson, 1998] or [Swanson, 2004]), the nomenclature for prefrontal cortex regions varied in research.

Humans and mice diverged around 100 million years ago. A lot can change in this time frame. As it will be discussed in the next section, mice can survive without a fully developed neocortex.

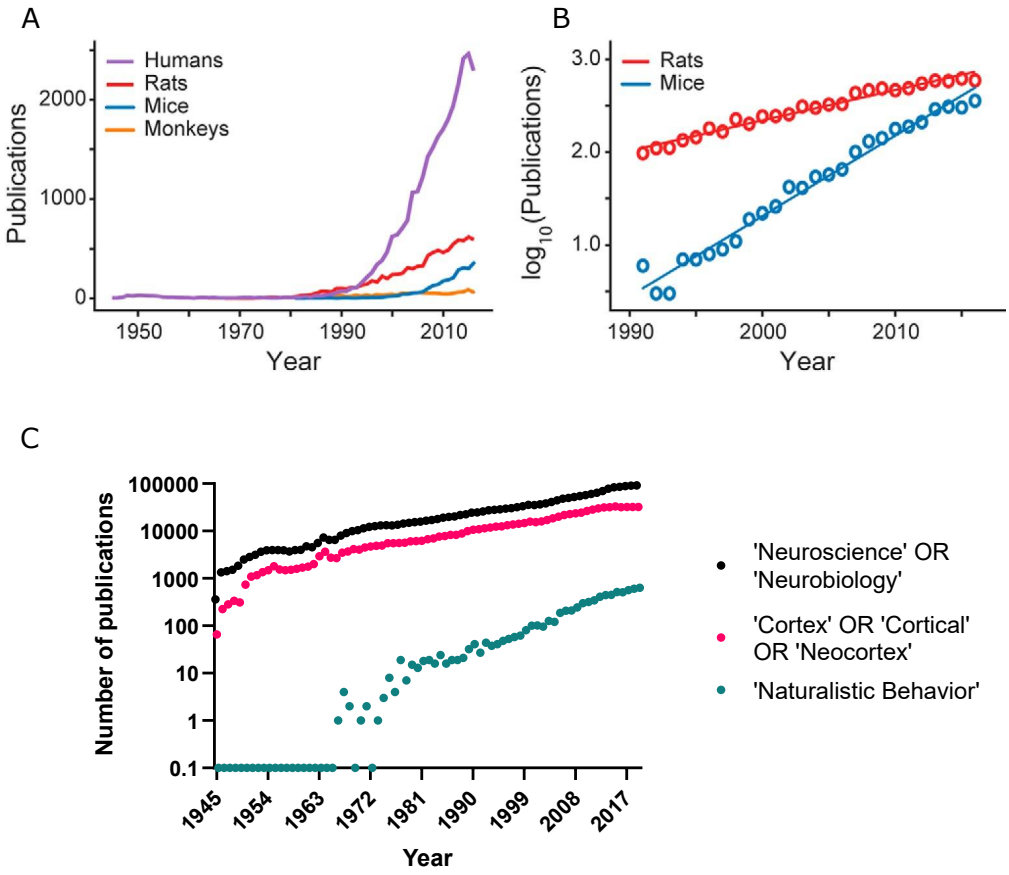

Figure 1.1: Publication records in neuroscience emphasizing the significance of cortex. (A) Publications on the prefrontal cortex of humans, rats, mice, and monkeys from 1945 to 2016. (B) Records showing that the rate of publications is accelerating more for mice relative to the rate of publications in rats. (Figures from [41]). (C) PubMed records of all research articles with three search queries between 1945 and 2019. Results of query for "Cortex" OR "Cortical" OR "Neocortex" follows the same trend as the search query results for "Neuroscience" OR "Neurobiology."

1.4 Mice Without Cerebral Cortex and Hippocampus

The main method for generating acortical mice for my thesis work was by utilizing a previously described mutant mouse model [34]. Apical complex protein, Pals1, was shown to be necessary for the proper cell migration and survival of cortical progenitor cells during embryonic development. The full knockout of the Pals1 gene in cortical progenitor cells was lethal. Researchers were able to create a knockout mouse model by deleting exon 3 from the Pals1 gene via Cre-mediated recombination in the Emx1 expressing cortical progenitor cells.

Pals1 flox/flox mice (gift from Dr. Seonhee Kim and Christopher Walsh) were bred

with Emx1-Cre (JAX Mice:005628) to create the acortical mutant mice. The effect of this mutation is dose-dependent. If only one copy of the Pals1 gene is removed, the resulting heterozygous progeny has some neocortical and hippocampal tissue spared. The homozygous genotype with both copies of Pals1 affected resulted in the animal we wanted to test: a mouse which lacks its neocortex and hippocampus, but retained the subcortical circuits (Figure 1.2).

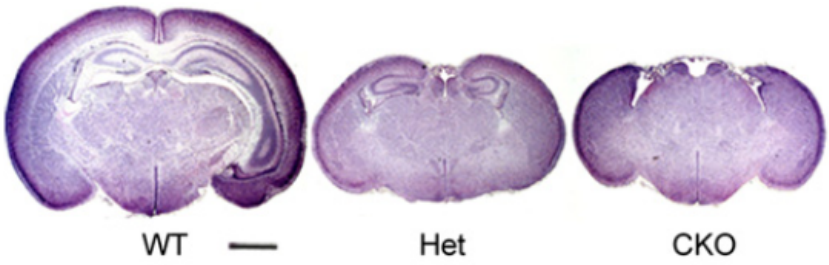

Figure 1.2: Homozygous mutant mouse lacking neocortical and hippocampal structures. Coronal sections from wildtype (wt), heterozygous (het), and homozygous (CKO) mutant mice (Figure from [34]. Scale bar, 1mm. See Appendix: TissueCyte Reference for all coronal sections from wildtype, heterozygous, and homozygous animals.

The original study concluded that due to lack of the visual cortex and other association areas, the homozygous acortical mice do not have intact vision. Their conclusion was based on the results of forepaw reaching test and Morris Water Maze experiments. Morris Water Maze is an experimental setup to normally assess spatial learning and memory [68]. Following training, an animal is dropped in opaque liquid and is expected to locate and navigate (swim) to a platform based on distal visual cues on the walls of the tank. Therefore, the results of this test can be used to infer deficits in navigation, learning and vision. Other deficits were also reported such as lower birthweight and low performance in wire hang test.

This mouse model is not the only acortical mutant that has been reported. In fact, a very similar model was shown to generate acortical mice that can perform normal ultrasonic vocalizations [27], suggesting that acortical mutants might be able to perform natural behaviors, but not necessarily the ones that are constructed in laboratory setting and do not reflect the conditions encountered in nature.

Chapter 2

STRUCTURAL AND FUNCTIONAL ASSESSMENT OF SUBCORTICAL STRUCTURES IN ACORTICAL MICE

For more than a century, scientists have stimulated or eliminated specific brain regions in an effort to understand their functional role. Most notably, the decorticated cats, dogs, and rats led to ideas such as distributed memory [39], sham rage [3], and cortical inhibition of fear. The methods relied on surgically removing the cortical areas or performing surgical interventions to inactivate specific regions of the cortex. Using a structural mutant mouse has many advantages. First, the genetic knockout model does not require any external intervention since the genetic manipulation takes place during embryonic development. Second, the effect of this manipulation will be similar across all animals that share the same genotype therefore potentially decreasing any variability that might be observed with other interventions. Third, practically an unlimited number of test subjects can be generated as long as the breeding colonies are maintained. We also attempted to create a similar effect by removing the neocortex and dorsal hippocampus surgically. The animals survived, but suffered blood loss, tissue damage, and inflammation. These consequences as well as the variations in surgical outcomes may lead to unintended consequences and manifest increased variability during behavioral experiments.

Using a structural mutant comes with its challenges. The conditional knockout of the gene Pals1 during the embryonic development may lead to consequences other than the missing neocortical and hippocampal tissue. The following experiments were conducted to validate the structure and function of the subcortical circuits.

2.1 Morphological Validation Experiments

First, we focused on the attention and visual processing centers within the subcortical circuits. The retinotectal pathway is evolutionarily conserved. The retinal ganglion cells send direct projections to the superficial layers of superior colliculus (SC), and along with the periaqudectal gray (PAG), these subcortical regions are implicated in the sensorimotor transformation and motor outputs in visually guided behaviors [10]. The coronal sections from wildtype and mutant mice clearly show the lack of neocortex and hippocampus. However, assessing the conservation of SC in the acortical mutant mice is not straightforward.

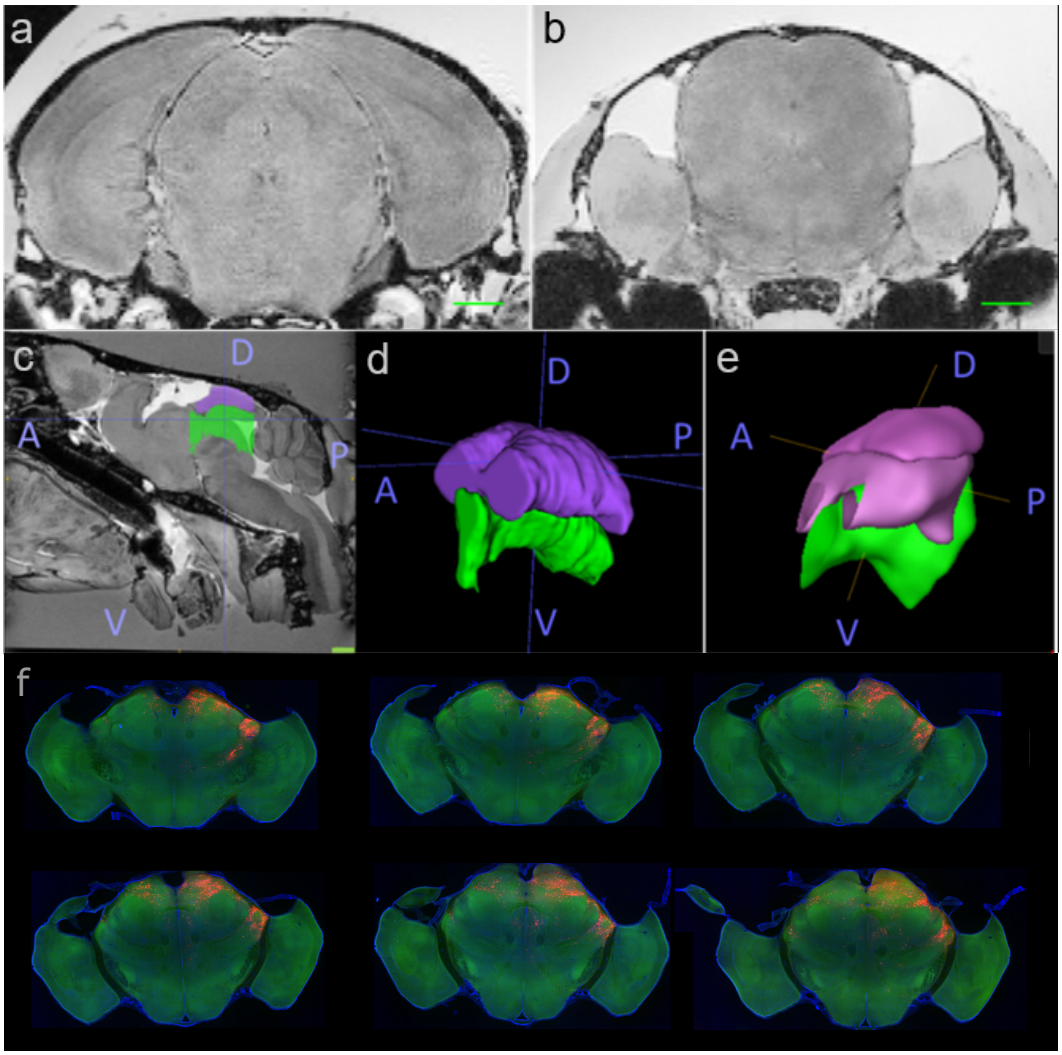

Figure 2.1: Midbrain structures conserved in the mutant mouse. Superior colliculus in the mutant mouse does not receive cortical input. High resolution T2*-weighted 3D gradient echo images of (A) wildtype and (B) mutant mice. (C) Sagittal section of mutant mice showing superior colliculus (SC) in purple and periaqudectal gray (PAG) in green (C). (D) 3D reconstruction of SC and PAG in mutant and (E) wildtype mice. Scale bar, 1mm. (F) Mutant mice lack any cortical input to SC identified via long-term HSV-hEF1-mCherry (MIT Vector Core) injections in superficial SC for retrograde labeling of projection neurons. Scale bar, 1mm.

Due to the nature of the genetic manipulation, the homozygous mutant mouse never developed the cortical and hippocampal structures. As a result, the skull of the mutant mice is flat when compared to the skull of wildtype mice. The flat skull caused a slight change in the shape of the dorsal part of superior colliculus in the mutant animals, preventing proper alignment for comparison of individual coronal sections of the mutant brain to the coronal sections of wildtype brain. We first

obtained MRI scans of wildytpe and mutant mice brains while still in the skull. 3D gradient echo sequences were used for 3D reconstruction of SC and PAG. The volume of superior colliculus in mutant mouse was 9.4mm^3 (Figure 2.1, C & D) which is comparable to the volumes reported in wildtype animals [2] whereas the neocortex and hippocampus are missing in these structural scans (Figure 2.1, B).

In wildtype animals, primary visual cortex (V1) sends direct projections to the superficial layers of SC. Using the retrogradely transported long-term non-transsynaptic virus, HSV-hEF1-mCherry, we confirmed these projections in the wildtype animal. If the acortical mutant mouse is truly missing all neocortical inputs, injection of the long-term HSV-hEF1-mCherry virus should not result in any soma labeling in the remaining ventrolateral tissue. As expected, we did not observe any projection neurons from the remaining ventrolateral tissue in the mutant mice (Figure 2.1, F). This result suggests that the full knockout acortical mutant does not have any primary visual cortex. We observed projections to the injection site from other subcortical regions which suggested that the subcortical wiring during development might have been unaltered.

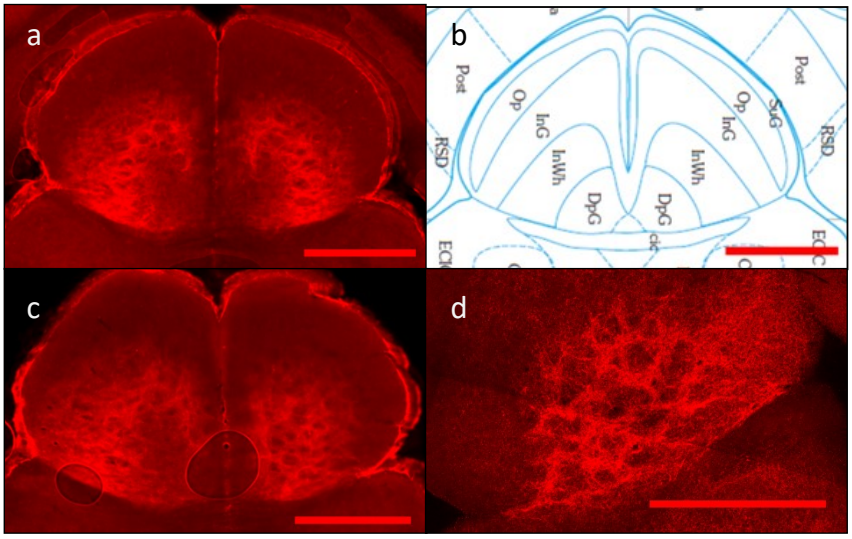

Figure 2.2: Cholinergic projections to superior colliculus are conserved in mutant mice. (A) Modules within the intermediate layers of superior colliculus stained with vesicular acetycholine transporter antibody. (B) C57BL6/J horizontal section showing the modular structure formed by cholinergic inputs to intermediate SC, corresponding horizontal atlas section. (C) Modular structure is conserved in the heteroyzgous acortical animal. (D) The honeycomb structure is better observed in tangential sections (maximum Z-projection of section from wildtype animal). Scale bar 1mm.

Additionally, we checked for conserved connections within the remaining subcortical circuits. The parabigeminal nucleus sends cholinergic projections which innervate the intermediate layers of superior colliculus [47]. When visualized in horizontal or tangential sections, these projections form a lattice, specifically a honeycomb structure (Figure 2.2, A & D). These projections have been confirmed in rodents [55] as well as feline species [26]. We found that the acortical mice had the same honeycomb structures conserved (Figure 2.2, C), suggesting that the development of subcortical circuits were not perturbed by the conditional knockout of Pals1 gene.

2.2 Functional Validation Experiments

Conservation of structures and projection patterns within the subcortical regions of the mutant mice is reassuring. However, structural similarities do not certify that the function of these circuits are conserved. Since the main sensory modality that will be challenged in behavioral experiments is vision, we looked at the visual processing similarities between the wildtype and mutant animals.

In a head-fixed setup, it is possible to perform extracellular recordings from the superficial and deeper layers of superior colliculus simultaneously as the animal is presented different visual stimuli within the receptive field of the recorded cells. Previous work has shown that the superficial layers of SC have neurons that respond reliably, without adaptation to multiple presentations of the threat stimulus: an expanding dark disk (Figure 2.3, A). During the same recordings, neurons were found within the deep layers of the SC which would increase their firing rate in response to only the first threat stimulus, but not during the subsequent presentations. Remarkably, the same type of responses were recorded in the superficial and deep layers of SC in the mutant mice (Figure 2.3, B). These results suggest the following: (1) the retinotectal pathway in mutant animal is functionally conserved, and (2) the sensory processing in response to visual threat stimulus is conserved throughout the different layers of the superior colliculus in the mutant mice.

In support of the conserved functionality within the retionotectal pathway of the mutant mice, we also showed that a subset of neurons in superficial layers SC can be identified using activity-dependent immediate-early-gene labeling following visual threat exposure in a head-fix experimental setup both in wildtype and mutant mice (See Appendix: Activity-dependent Labeling in sSC).

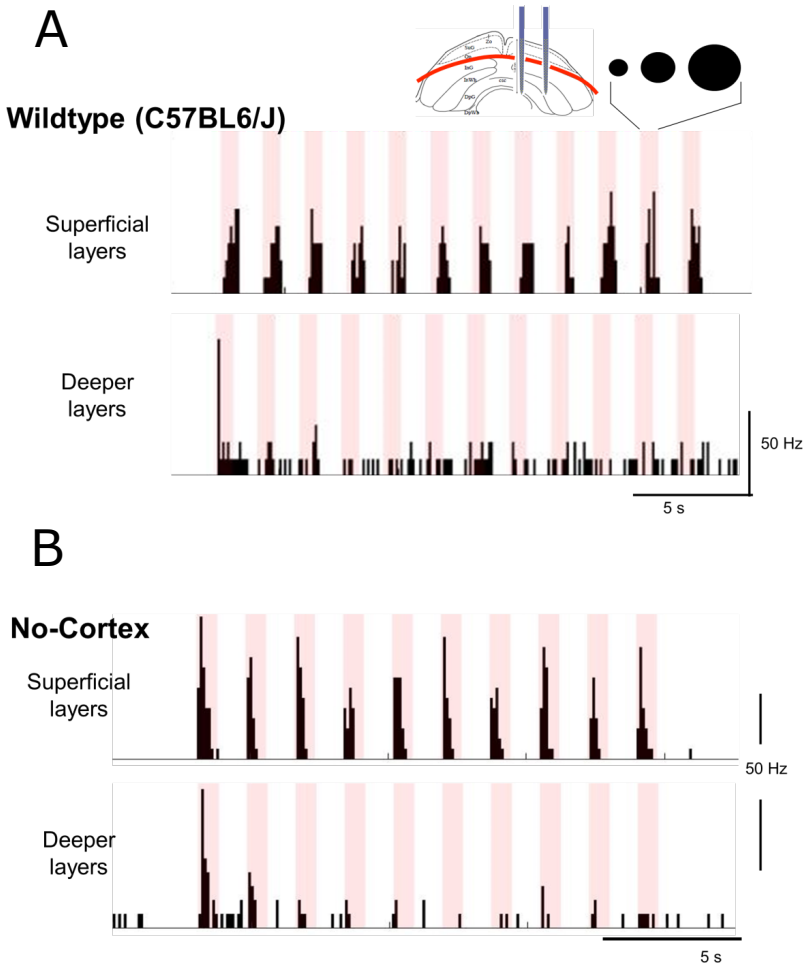

Figure 2.3: Extracellular recordings of neurons in mouse superior colliculus. (A) Multi-electrode probes were used for recording the neuronal activity in awake head-fixed mice. Neurons in the superficial layers of SC in wildtype mice exhibited increased firing rates in response to each presentation of a black expanding disk (pink bars). In contrast, rapid habituation was observed in neurons within the deep layers of SC following a single presentation of the looming stimulus. (B) Same patterns of neuronal activity were observed in the mutant mouse in both superficial and deeper layers of SC. Figure adapted from [42].

2.3 Alternative Circuit Interventions

In this section, we will go over methods that were considered, tested, and applied for the removal of neocortical and hippocampal structures.

Reversible Manipulations: Drug Infusions, Chemogenetic and Optogenetic Silencing of Neocortex and Hippocampus

Reversible manipulations are powerful tools for investigating neural circuits by temporarily modifying their function. Although drug infusions have been a hallmark of *ex vivo* electrophysiology studies, it is possible to use these drugs by chronic implant of a cannula through the skull and brain tissue. The limitation of this method for our use is that only a specific brain region can be targeted for *in vivo* drug infusions. Muscimol, a selective agonist for the GABA subtype A receptors, has been used extensively and would have been the ideal candidate if our target region was more localized. Due to the same reason, optogenetic activation of GABAergic interneurons or silencing of excitatory projection neurons throughout the neocortex would not be feasible. Chemogenetic manipulation following the same logic might have allowed us to express inhibitory DREADDs throughout the Emx1 expressing neurons in a Cre-mediated pattern. However, due to the systemic expression of these effectors, we would not be able to rule out non-neuronal causes for changes in behavior.

Irreversible Manipulations: Targeted Cell Ablation using Viral or Toxin-based Methods, Excitotoxic Lesions, Tissue Aspiration

We tested multiple irreversible techniques to create alternatives to the structural mutant mice. Selective ablation using diptheria toxin subunit A expression in the cortical progenitor cells during development was lethal to all progeny. We then tried a different method using similar tools: We first expressed the diptheria toxin receptor in Emx1 expressing cells, followed by injection of diptheria toxin subunit A once the progeny was born. No litter survived the DTa injection.

Another method that is widely used for localized lesions is injecting agonists of glutamate receptors in a specific brain region. These agonists lead to over-stimulation of the neurons causing excitotoxicity. Using ibotenic acid, an agonist of glutamate receptors, we made several attempts to create extensive excitotoxic lesions of the dorsal cortex and hippocampus. However, the subjects did not survive the post-surgery seizures.

Surgical removal of brain tissue has a long history in animal behavior and neuroscience research [21, 38, 40] . We were able to remove neocortical and hippocampal tissue surgically. We performed bilateral craniotomies (diameter 3mm) and aspirated the dorsal cortex and hippocampus. The animals that survived the tissue

aspiration were tested in predator avoidance and labyrinth experiments.

An alternative method of caspase-mediated cell ablation of Emx1+ neurons was not tried. This method leads to apoptosis in genetically identified cell population and has been used successfully in subcortical regions previously [37, 73]. Given the extensive coverage necessary for this method to eliminate all neocortical and hippocampal structures, future studies should try using genetically engineered viral vectors for noninvasive delivery of caspase 3 via neonatal injection in Emx1-Cre mice [9].

Chapter 3

PREDATOR AVOIDANCE IN WILDTYPE AND ACORTICAL MICE

3.1 Background and Motivation

Predator avoidance is crucial for survival. Mice rely on their vision to avoid an attack from an aerial predator. The image of an approaching predator hits the retina of the mouse, and the mouse has to exhibit a suitable defensive behavior to ensure its survival. The time it takes from the moment of detection of the approaching predator to the moment of a motor response is a very fast process (Figure 3.3, D). Given the speed of the sensorimotor transformation and the conservation of the behavior in non-mammalian vertebrates, the evolutionarily ancient brain region, optic tectum (superior colliculus in mammals) is thought to be the main brain region involved in regulating this innate defensive circuit. Superior colliculus is a multi-layered midbrain region which receives direct input from the retinal ganglion cells in its superficial layers.

Innate defensive behaviors of escape and freezing have been shown in laboratory setting by placing the animals in a small arena and presenting an expanding dark disk on a monitor above the animal [74]. Previous work has shown that wildtype animals mostly either escape or freeze in response to the black looming disk stimulus which occupied up to 20 ° of the animal's upper visual field. The animals appropriately did not respond to the same stimulus if presented on the side of the arena or from the lower visual field. Behaviorally irrelevant visual stimuli, such as bright expanding or bright receding disk, did not elicit frequent escape responses and mostly resulted in freezing or rearing in response to innocuous stimuli.

This behavioral paradigm is ideal for testing the capacity of subcortical circuits in naturalistic behaviors which are necessary for survival. We challenged the wildtype, acortical mutant and lesioned mice in the predator avoidance assay.

3.2 Behavioral Responses of Wildtype, Acortical Mutant, and Lesioned Mice

The black looming stimulus used in the predator avoidance assay mimics the shadow of an approaching predator (Figure 3.1, A). In these experiments, we first optimized the arena. Using infrared transmitting black acrylic plastic, we designed a large

arena blocking all visual distractions from outside the arena and giving the animal more space to explore making the environment more like an open field. We also used a very small shelter (<3x the size of the mouse) with a small opening resembling a small burrow or nest. The maximum size of the expanding dark disk covering the upper visual field of the mouse was increased to 50°. The animals were group housed to reduce the likelihood of observing fear behaviors due to social isolation [75].

A single animal was placed inside the cage and was allowed to explore (Figure 3.1, B). The visual threat stimulus was not presented until the animal explored the inside of the shelter during the acclimation period. Ten consecutive dark expanding disks were presented in the upper visual field of the animal on a monitor with 1 second inter-stimulus-intervals (ISI). The typical response of wildtype animals consisted of a robust escape directed towards the shelter (Figure 3.1, C. Note that the trace is pink when the animal is inside the nest). Some wildtype mice initially exhibited short freezing bouts, but always escaped to shelter during the stimulus presentation.

Acortical mutants, similar to the wildtype mice, showed robust escape response to the visual threat stimulus except for one acortical mutant mouse which showed freezing response throughout the stimulus presentation (Figure 3.2). Surprisingly, the acortical mutants and the lesioned mice reacted with consecutive escape bouts for subsequent presentation of the stimulus and did not show any habituation or change defensive strategies to the ten repetitions of the looming stimulus.

We did not observe any repetitive escape bouts in the wildtype animals, but this might be due to their directed escape to the shelter during the stimulus presentation (Figure 3.3, C). Once inside the shelter, the visual threat detection circuit is no longer activated. Would the wildtype mice exhibit such robust and repetitive escape responses similar to the lesioned and mutant mice, if there is no shelter available? To answer this question, we tested a group of naïve wildtype animals with the same visual stimulus and experimental conditions except for the availability of the shelter. The wildtype mice exhibited a few escape bouts, but exhibited mostly freezing behavior during the visual threat presentation.

When we quantify the number of escapes per trial, the acortical mutants and lesioned mice had significantly more escape bouts compared to wildtype animals, even when a shelter was not present (Figure 3.3, C). This result suggests that cortex might be providing a switch mechanism out of the escape mode and into the freezing mode during stimulus presentation in wildtype mice. Without the cortical input,

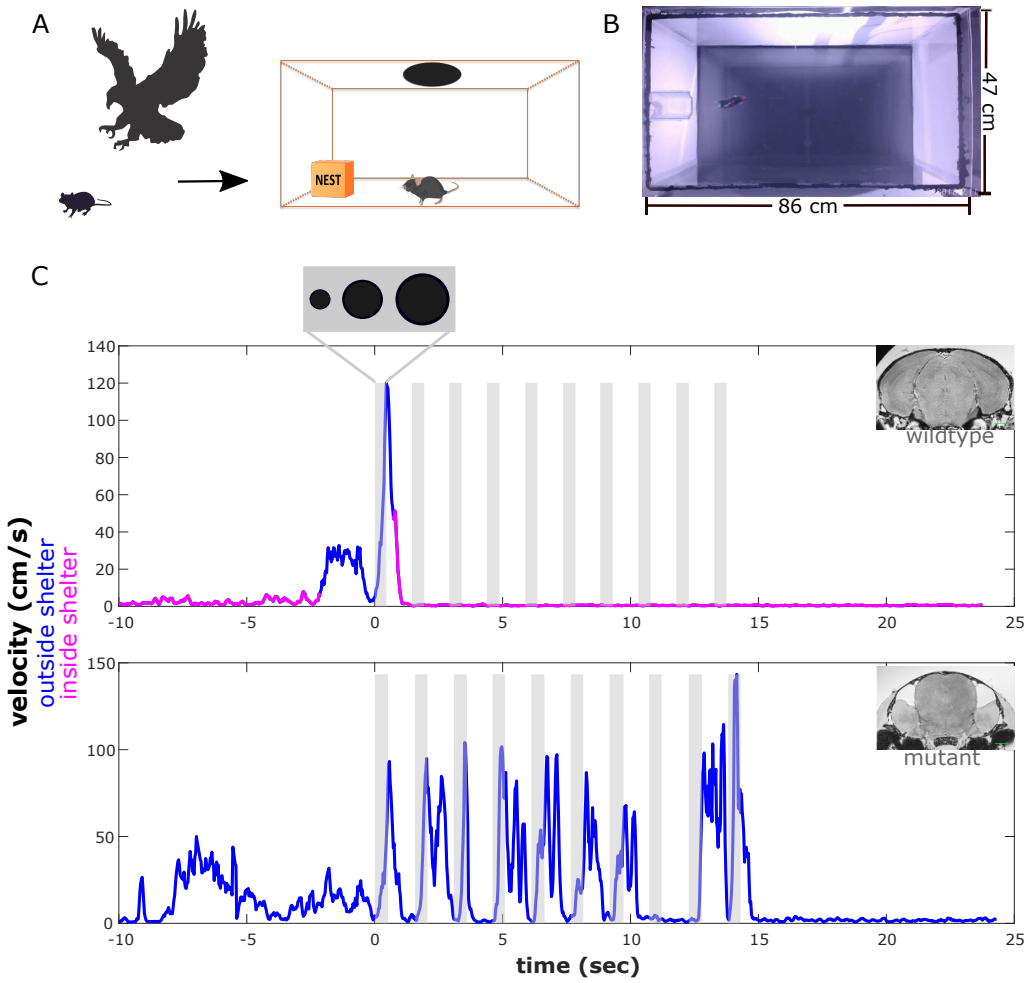

Figure 3.1: Predator avoidance behaviors of wildtype and mutant animals in the laboratory setting. (A) Schematics of looming assay: Expanding dark disk is presented on a monitor above a large arena. (B) The arena (86cm L x 47cm W x 30cm H) was made of infrared-transmitting black acrylic. Video recording from below the arena allowed for accurate tracking using DeepLabCut [50]. (C) Representative velocity plots from a wildtype (top) and a mutant (bottom) mouse showing escape behaviors in response to the visual threats presented. Each gray bar represents a single black expanding disk. The velocity trace is blue when the animal is outside the nest/shelter, pink when the animal is inside the shelter.

the animals are behaving in a reflexive manner. The perceived hyper-reactivity in acortical mutants and lesioned mice might be due to abnormalities in locomotion leading to an overall increased velocity. We quantified the velocities before any visual stimulus presentation to rule out this possibility (Figure 3.3, B). There was no significant difference in velocities among the groups of animals tested prior to visual stimulus presentation.

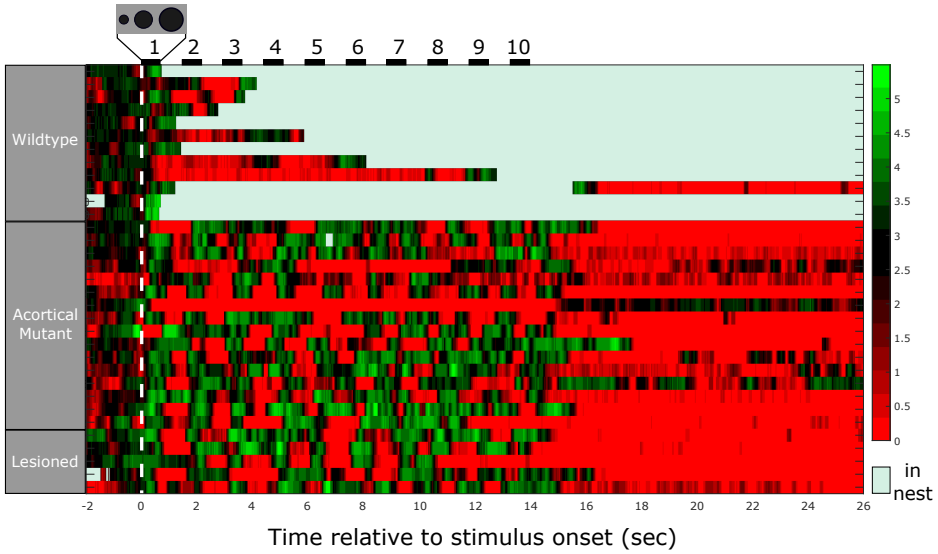

Figure 3.2: Wildtype, acortical mutant, and lesioned animals exhibit intact innate defensive behaviors in response to visual threat stimulus. Mutant and lesioned mice show robust and persistent escape bouts and impaired shelter-directed navigation. Velocity heatmap showing individual animals' responses to 10 consecutive black looming disks with 1 sec ISI. Escape bouts indicated in green, freezing in red, animal inside shelter in light blue.

Without any cortical intervention, are subcortical circuits responding more robustly to visual stimuli? Does a subcortical circuit complete the sensorimotor transformation faster than the one that receives input from the cortex? To answer these questions, we also quantified the latency to respond in each group. The time it took for the animal to react was assessed visually by the experimenter. The reaction is defined as visible lowering of body and increased muscle tension. The latency to respond did not differ significantly among all groups tested (Figure 3.3, D).

3.3 Directed Escape and Shelter-Seeking Behaviors

The wildtype animal behavior in response to visual threat stimulus was consistent across all animals tested. All mice ended up in the shelter via directed escape even if the latency to escape was slightly different. Among the acortical mutant and lesioned mice tested, no animal made a directed escape to the shelter during visual threat presentation. In fact, even if an animal encountered the inside of the shelter during an escape bout, the animal continued the escape without stopping for refuge. Contextual and stimulus parameters were changed to induce directed escape in the mice that lacked their neocortex and hippocampus.

Acclimation

The animal was allowed to acclimate in the arena for 22 hours with excess food and water. To assess the effect of the increased acclimation period, water and food were placed in different corners of the arena on the opposite side of the shelter to prevent any positive association. This acclimation period allowed for extensive exploration of the arena and the shelter.

Positive Reinforcement

The animal was water-deprived for 23 hours in the home cage. A container filled with water was placed inside the shelter. The animal was then placed in the arena and was given time to explore. Once the animal consumed some water inside the shelter and continued to explore the arena, the visual threat was presented.

Shelter

The mice were acclimated to the arena with a T-shaped tunnel-like shelter. This shape and size resembles a more naturalistic burrow that the animals might have access to under naturalistic conditions. Given the shape, this shelter also allowed two entry points and covered more surface area of the arena compared to any other shelter type.

These variations and adjustments did not alter the behavior of the acortical mutants in response to the visual threat stimuli: all tested animals exhibited robust escape bouts. Directed escape to the shelter was not observed under any of these conditions.

3.4 Stimulus Discrimination in Acortical Mice

A common theory regarding the role of cortex is that it provides inhibition for the subcortically generated fear responses. This theory is supported further by the 1930s experiments that resulted in "sham rage" in cats and dogs that were decerebrated [3]. These results would suggest that the acortical mutants and the lesioned animals would be exhibiting escape behaviors in response to behaviorally irrelevant, innocuous stimuli which had similar parameters to the threat stimulus. To test these assumptions, we used a bright receding disk stimulus: a white disk shrinking at the same speed as the black looming disk, starting at 50 $^\circ$ in the upper visual field of the mouse. Both stimuli provide a moving dark edge and cause a decrease in luminance in the animal's upper visual field. White receding disk does not, however, have any resemblance to a naturalistic stimuli. Our results showed that all animal groups (wildtype, acortical mutant, and lesioned mice) responded with

decreased mobility and did not exhibit robust escape responses to the consecutive presentation of the receding disks (Figure 3.4). We did not find any significant differences between the three groups of mice tested in response to the white receding disk stimuli (Figure 3.5).

Another control stimulus tested using the three groups of mice was the white looming disk. This visual stimulus is again not a behaviorally relevant one, but can elicit escape and freezing responses as reported previously [74]. Similar to our results from the white receding disk stimuli experiments, we did not observe any repetitive escape bouts in response to white looming disk in acortical mice (Figure 3.6). The main distinction between the threatening black looming disk stimuli and the innocuous visual stimuli has been the shelter-seeking behavior in wildtype mice. In response to the black looming disk, all wildtype animals sought shelter whereas in response to the white receding and white looming disks, the wildtype mice did not escape to shelter. The stimulus discrimination in both the wildtype and acortical mice functions similarly. When exposed to a threat, the animal runs. When exposed to a non-threatening salient stimulus, the animal freezes.

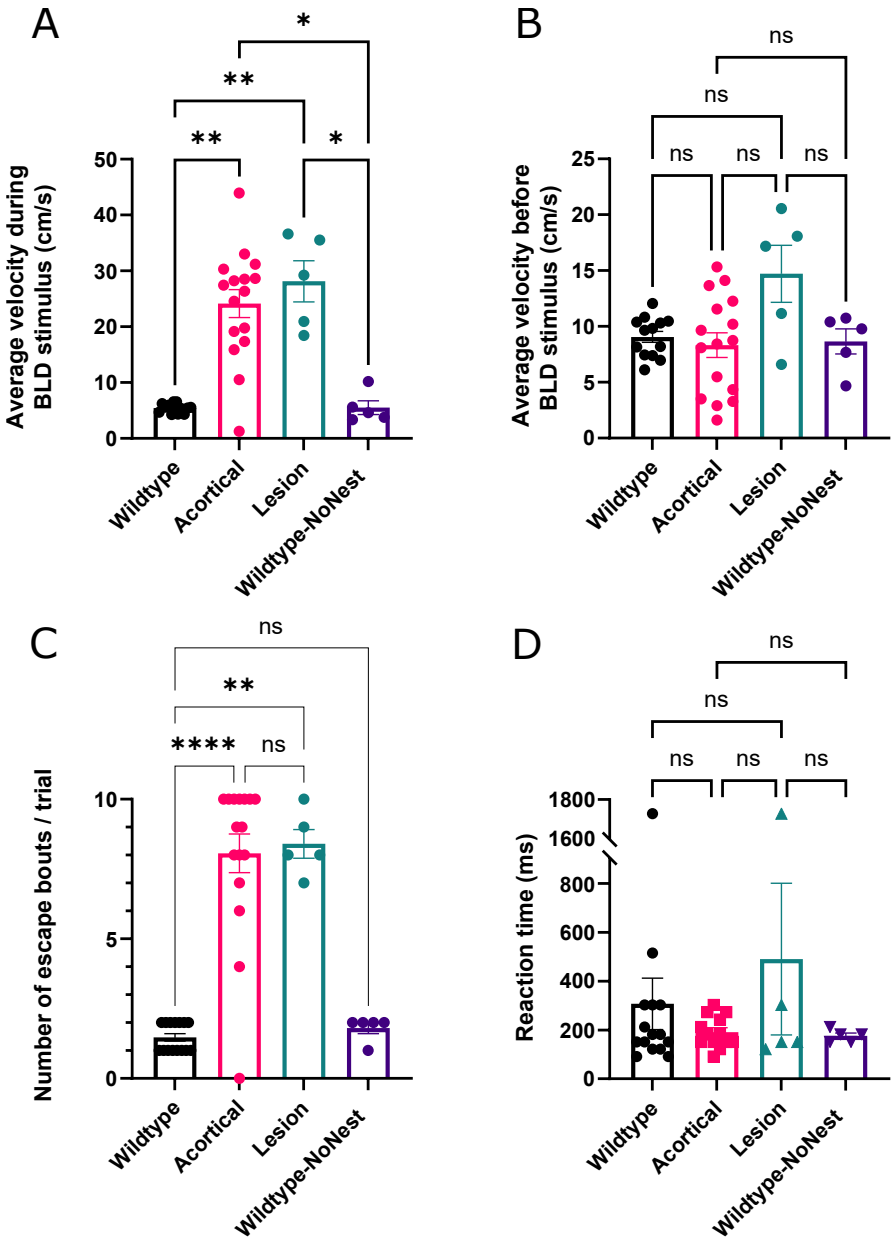

Figure 3.3: Persistent defensive behaviors of the mutant and lesioned mice. (A) Acortical animals were more responsive during threat stimulus presentation when compared to wildtype animals indicated by the average velocity during stimulus presentation. (B) This observation was not due to increased baseline locomotion of mutant and lesioned animals as all groups' average velocities prior to visual stimulation were similar. (C) Similar to the increased velocity during threat presentation, the mutant and lesioned animals exhibited more escape bouts in response to the 10 consecutive black looming disk stimulus per trial. (D) Latency to respond to visual stimulus remained the same for all groups. Kruskal-Wallis test for multiple comparisons; $\alpha = 0.05$.

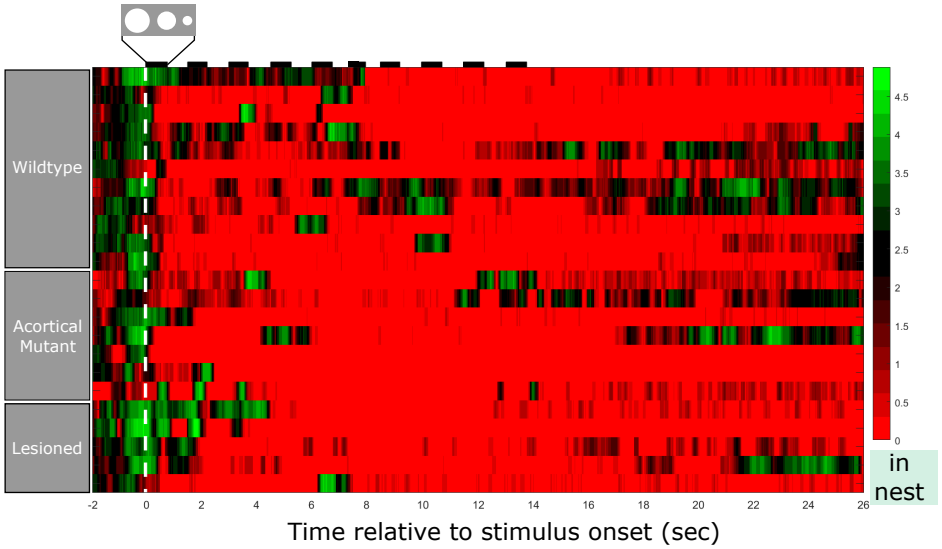

Figure 3.4: Behavioral responses of wildtype, mutant, and lesioned mice to innocuous visual stimuli: white receding disk. Acortical mutant and lesioned mice exhibit similar behavioral responses when compared to the wildtype animals in response to the behaviorally irrelevant, innocuous white receding disk stimuli.

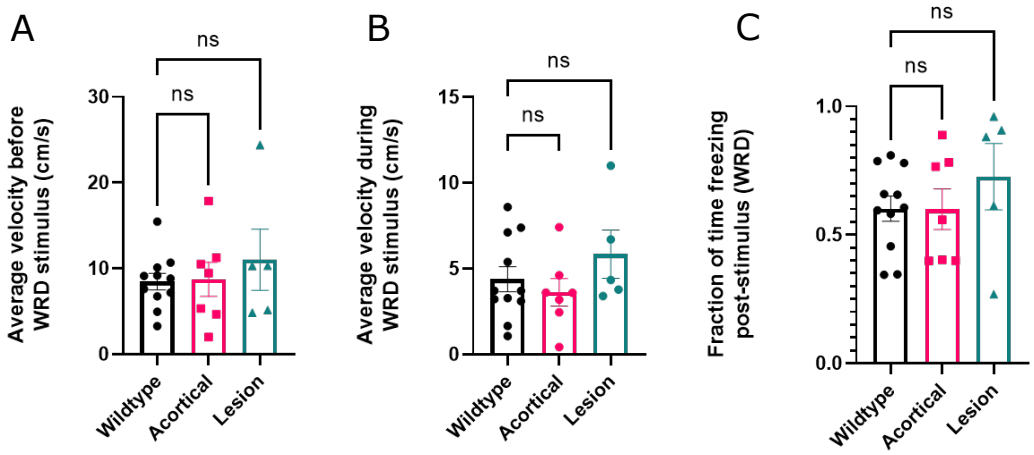

Figure 3.5: Wildtype, mutant, and lesioned animals respond similarly to the behaviorally irrelevant stimuli. No significant differences were observed before, during, or after the white receding disk stimuli presentation. (A) Pre-stimulus velocities were similar among all groups tested.(B) No robust and repetitive escape bouts observed in acortical mutant or lesioned mice. (C) Post-stimulus freezing was quantified as percentage of the time the animal was not moving during 1 minute following the stimulus offset.

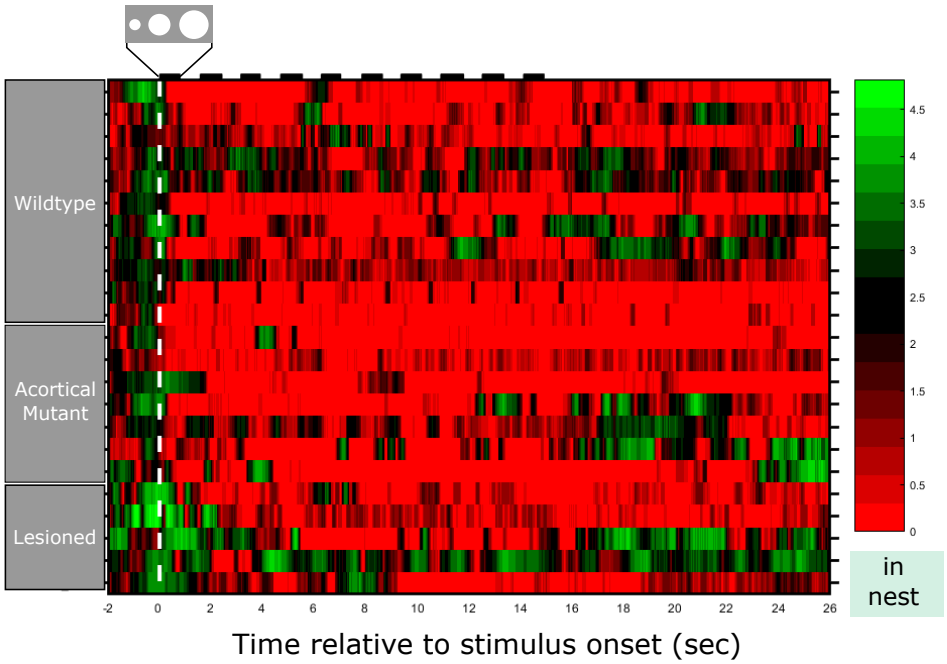

Figure 3.6: Behavioral responses of wildtype, mutant, and lesioned mice to innocuous visual stimuli: white looming disk. In response to another behaviorally-irrelevant stimulus, a white looming disk, shelter-seeking behaviors and directed escapes are absent in wildtype, acortical mutant, and lesioned mice.

Chapter 4

NAVIGATION AND LEARNING: MICE IN A LABYRINTH

4.1 Background and Motivation

For more than a century, neuroscientists and psychologists have placed rodents in mazes with the hopes of understanding learning and memory [22, 66]. More contemporary studies have focused on using empty arenas or linear tracks to analyze the activity of neurons involved in navigation in rodents [5, 35]. In an effort to understand how an animal learns to navigate in a novel environment, wildtype mice were recently tested in a complex labyrinth [60]. The 6-level binary maze experimental setup used in this study provided us the perfect opportunity to test whether acortical animals are capable of spatial learning (Figure 4.1). Here, we present our results for acortical mutants and lesioned animals which were tested in the same labyrinth under the same conditions as the wildtype animals. The wildtype data for comparison in this section is a subset of the animals described in "Mice in a labyrinth: Rapid learning, sudden insight, and efficient exploration" [60].

4.2 Acortical and Lesioned Mice in a Labyrinth

The acortical mutants and lesioned mice failed to locate the shelter under threatening conditions. One explanation would be the lack of hippocampus and medial entorhinal cortex, two brain regions that have been described to hold spatial information encoded in place cells and grid cells, respectively [54]. We tested the assumption that the acortical animals lacked the ability to navigate to a certain location by placing them in a complex maze. Following 24hr water deprivation, a single mouse is placed in a home cage attached to a complex labyrinth. The animal had free access to food in the home cage and could freely walk between the home cage and the maze. The animal needed to make 6 correct consecutive decisions to arrive at the water location inside the maze. However, the animal did not have any prior experience in this maze, thus had to explore until "running into" water. All experiments were completed in the dark using infrared transmitting black acrylic, reducing the likelihood that any visual cues could be used for learning and memory. Animal's behavior was recorded overnight along with the water reward information. All animals were given ~12hr to freely navigate the labyrinth and had access to the home cage. Only the time spent in the maze was recorded, and analyzed.

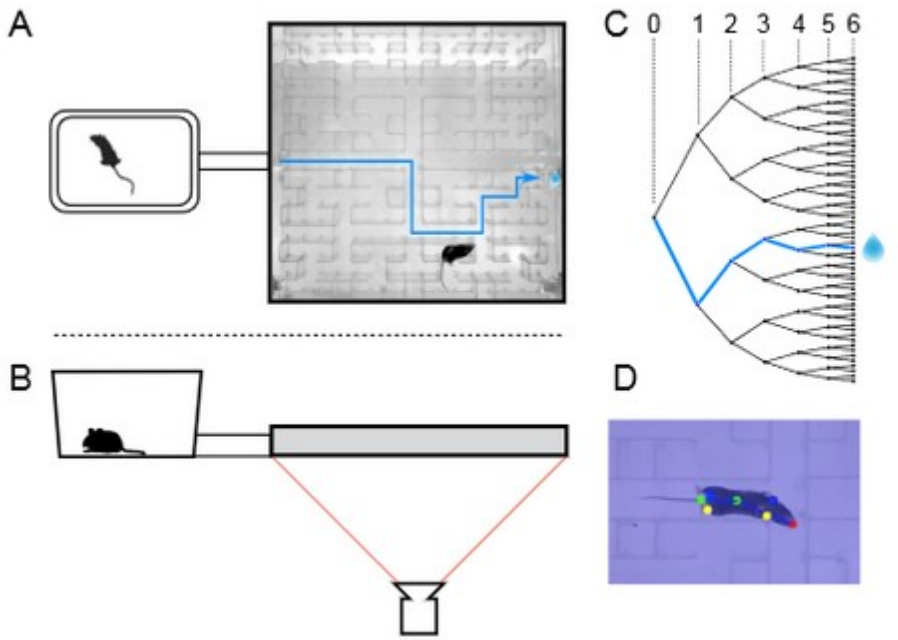

Figure 4.1: Navigation and goal-learning in a complex environment. Figure from [60] (A) The mouse is placed in the home cage which is attached to a 6-level binary labyrinth. (B) Activity of mouse in the labyrinth is recorded from below during the overnight experiment following 24hr water deprivation. (C) Six correct turns in a row will lead to the only water source in the labyrinth. Experimenter-defined body part information collected using DeepLabCut [50].

Our initial assumption was that the acortical mutants and lesioned mice lacked spatial information provided by the hippocampus and medial entorhinal cortex. This assumption was strengthened by our results from the predator avoidance assay as all the acortical mutants and lesioned mice failed to show directed escape to shelter. This assumption turned out to be incorrect. The acortical and lesioned animals were able to navigate in this maze, find the water location and collect water rewards following the discovery of the reward location (Figure 4.2). The time to collect the first water reward was longer for the acortical mice when compared to wildtype animals. We analyzed the velocity of the animals to check if this delay in water location discovery was due to inactivity (grooming or sleeping). Only one acortical mouse showed some bouts of inactivity (Figure 4.2, black lines). Unlike the wildtype animals, both the acortical mutants and lesioned mice spent almost all of their time inside the labyrinth during the first half of the experiment (Figure 4.3). We looked further into the exploration strategies of acortical mice during the

initial phases of the experiment to elucidate the reason behind the extended delay in discovering the water location in the labyrinth.

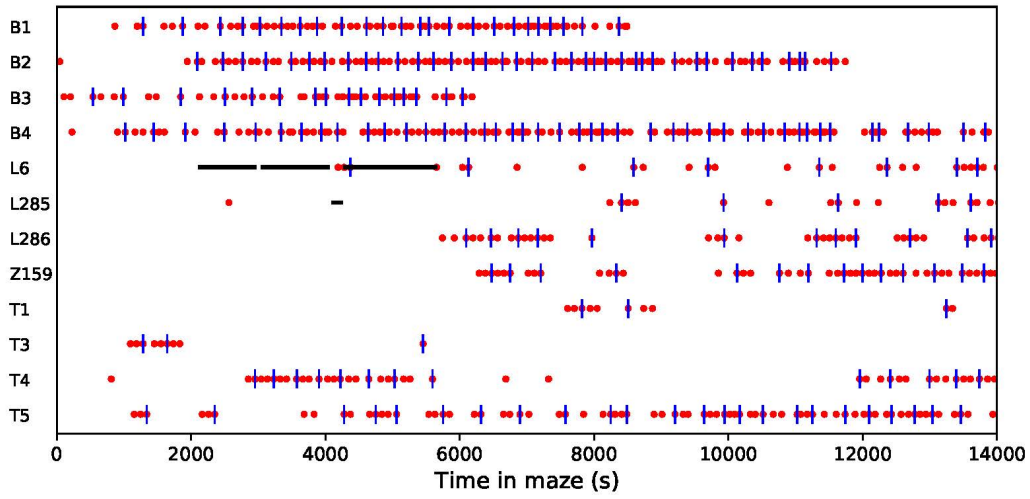

Figure 4.2: Timeline for water reward collection for wildtype and mutant mice. Red dots indicate each reward collected, blue ticks are placed once every 3 rewards. The black lines indicate when the animal is not moving for more than 2 minutes to assess locomotion during exploration. Wildtype animals (B1-B4, data from the Maze Team), acortical mutants (L6, L285, L286, Z159) and lesioned mice (T1, T3, T4, T5).

The abnormal initial exploration phase was observed in all acortical animals. During the initial phases, the acortical mice did not show any hesitation when entering the novel environment (Figure 4.4, B). Once inside the labyrinth, the acortical mice explored the labyrinth for a long time. This behavior was different in the wildtype mice (Figure 4.4, A). All but one wildytpe mice tested [60] exhibited hesitancy when entering the maze for the first time. The subsequent bouts of the wildtype animals involved more exploratory behaviors, visiting multiple end nodes within different quadrants of the labyrinth (Figure 4.4, A).

Under naturalistic conditions, mice navigate their environment efficiently [23]. In a complex labyrinth with 64 equally distant end nodes, how do we measure the efficiency of the animal's exploration? We defined efficiency of exploration as the number of distinct end nodes that the animal encountered based on the number of end nodes the animal visited. The efficiency of exploration of wildtype mice showed a slight decrease over the course of the experiment. The effect was opposite for the acortical mice (Figure 4.6). Although all acortical mice started navigating the maze in low efficiency (i.e. repetitively visiting the same end nodes within

the same small subspace without venturing out to other quadrants), throughout the course of the experiment, their efficiency of exploration increased. In fact, during the second half of the experiment, acortical mice showed the same level of efficiency in exploring the maze as the wildtype animals. Overall, these preliminary results suggest the existence of a cortex and hippocampus independent mode of spatial learning and goal-directed navigation. Future studies should test the wildtype mice and acortical mutants over multiple days to assess the differences in long-term memory. Future experiments can also focus on understanding the different sensory modalities involved in learning and navigation using this experimental paradigm.

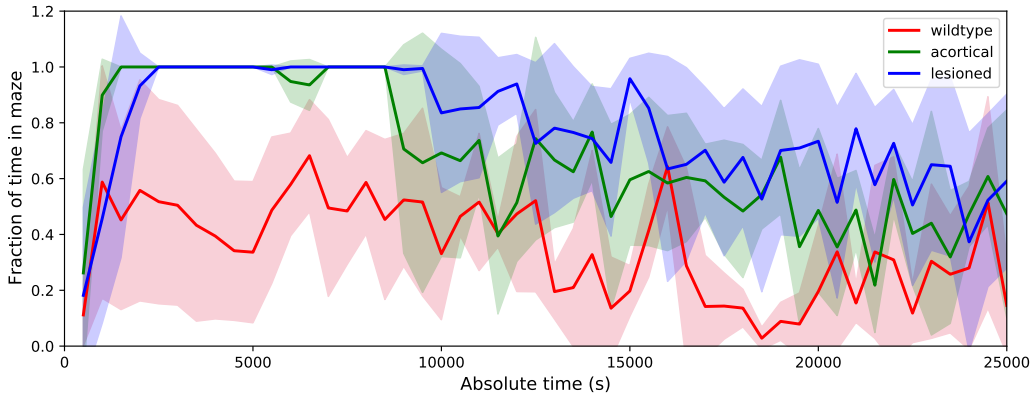

Figure 4.3: Mutant and lesioned mice spend more time in the labyrinth than the wildtype animals. Average fraction of time spent in maze by each group: wildtype mice (red), acortical mutant (green), and lesioned mice (blue). Mean ± SD over four animals tested in each group.

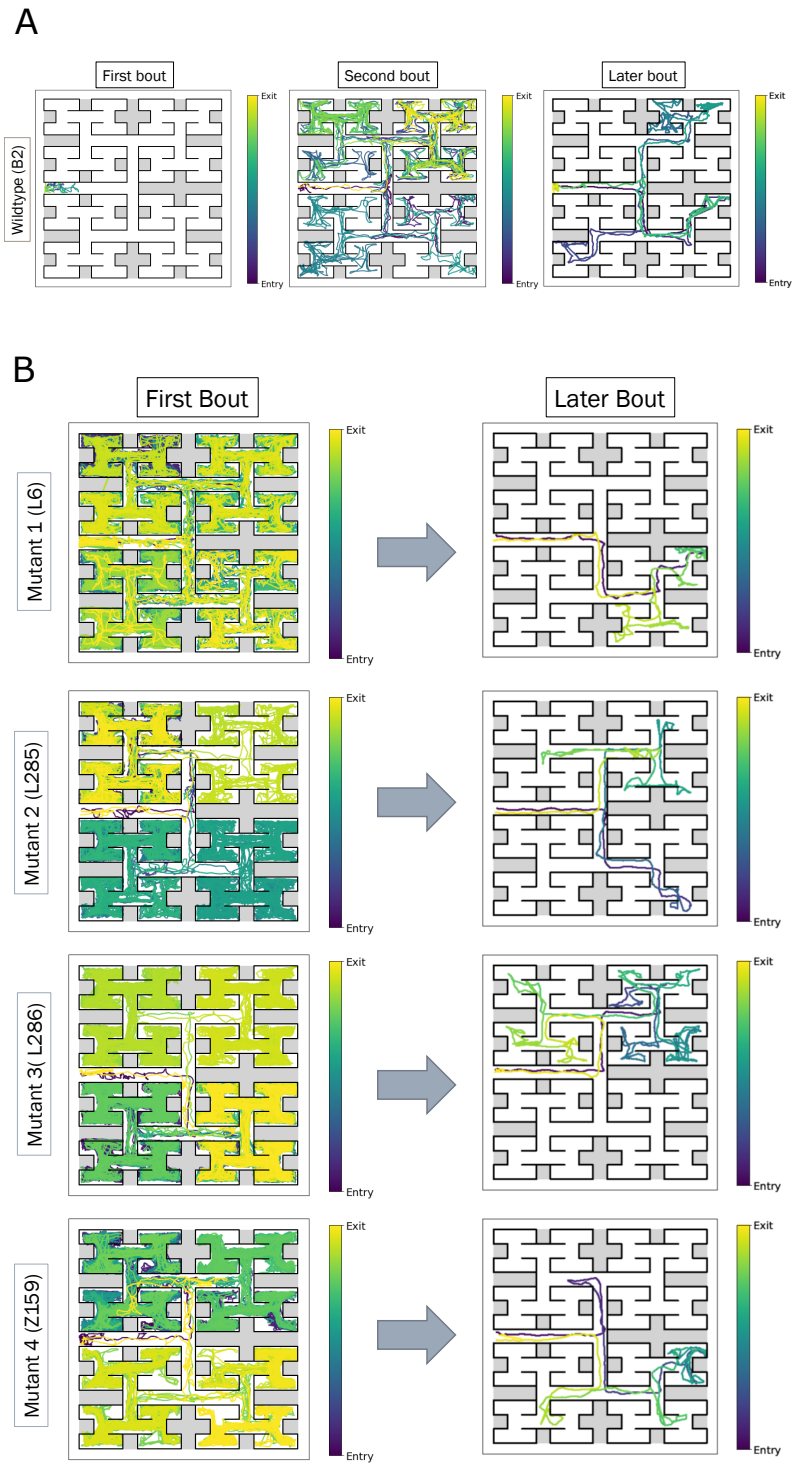

Figure 4.4: Exploration of novel environment in wildtype and mutant mice. (A) Wildtypes exhibit hesitancy during the first bout, start exploring during second bout, and show efficient exploration in later bouts. (B) Maze hesitancy was not observed in the acortical mutants. The mutants showed low efficiency exploration initially, but in later bouts an increase in efficiency of exploration was observed.

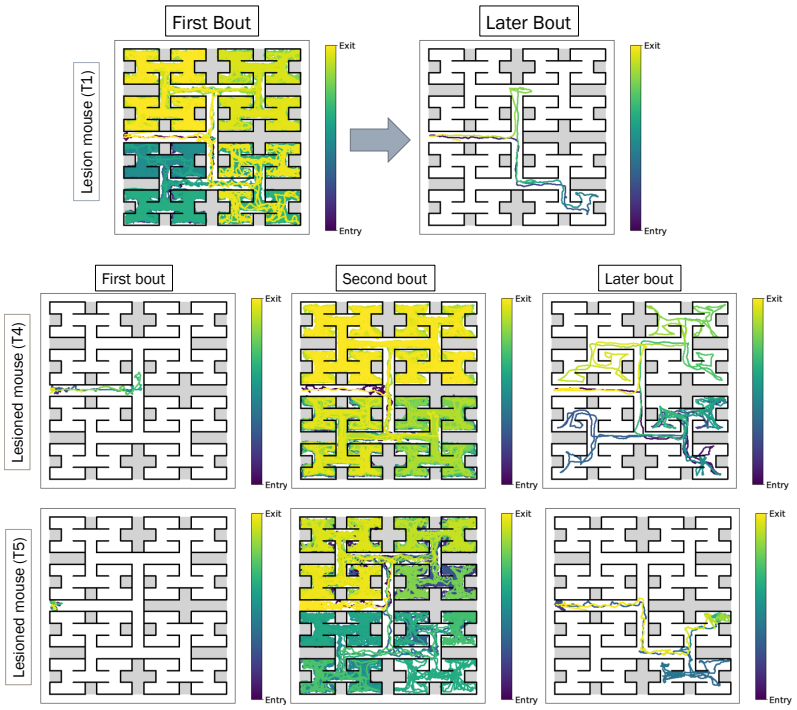

Figure 4.5: Exploration of novel environment in lesioned mice. Lesioned mice showed more variance in their exploration behavior exhibiting similarities to both wildtype and acortical mutants. Lesioned mice showed maze hesitancy during the first bout, but also exhibited the acortical mutant-like low efficiency of exploration initially which increased in the later bouts.

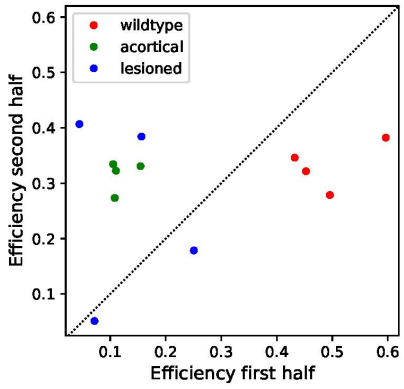

Figure 4.6: Efficiency of exploration changes throughout the duration of the labyrinth experiment. The time spent in the labyrinth was ~7hr for wildtype animals (red) [60]. Acortical mutant (green) and lesioned mice (blue) spent more time inside the maze than the wildtype animals (~12hrs).

Chapter 5

CONCLUDING REMARKS

5.1 Survival Without Cortex

We tested the capacity of subcortical circuits utilizing structural mutants and lesioned mice. The mutant and lesioned mice, similarly to the wildtypes, exhibit robust escape bouts to avoid approaching threat and freezing once the threat is no longer visible. However, the mutant and lesioned mice are unable to locate the shelter even when the animals had a chance to explore the shelter prior to the threat stimulus presentation, suggesting deficiencies in spatial learning and navigation when under immediate threat conditions. To test the hypothesis that a mouse without its neocortex and more importantly, its hippocampus, cannot navigate, we challenged the mice in a complex maze. We found significant differences in the learning rate, efficiency of exploration, and path integration. Compared to the control animals, the acortical mice took longer to explore the maze and locate the source of reward. After multiple successful excursions, the acortical mice were able to complete a perfect trajectory from their home cage to the reward location.

We found that the acortical mutants and lesioned mice can appropriately detect visual stimuli, discriminate between behaviorally relevant threatening stimuli and innocuous stimuli, and execute appropriate motor outputs based on the sensory input. The acortical mice, however, show deficits in directed navigation to a shelter when exposed to the looming stimulus and are resistant to adaptation in response to multiple exposures within the same trial. Surprisingly, when the mutant and lesioned animals were challenged with exploring a complex maze and locating a reward, all animals were capable of navigating the maze and learning the reward location.

This study provides new insights into the capacity of subcortical circuits by using a genetic mutant that fails to develop its neocortex and hippocampus. In addition to the conserved survival circuits, our results suggest a subcortical circuit for navigation and learning independent of the hippocampus and neocortex. Future studies, using this and other animal models, will provide insight to the capacity and capability of the ancient structures of the central nervous system.

5.2 Inferences on the Role of Cortex

The acortical mutant and lesioned mice behaviors in response to threatening visual stimuli allows us to conclude two things: (1) Visual processing of threat stimuli is intact in acortical mice. (2) Sensory-motor transformation circuit required for stimulus-dependent behavioral output is conserved within the subcortical structures. Although this work focused on what the subcortical circuits can accomplish independent of the neocortex and hippocampus, inevitably a complementary question arises. If the subcortical circuits have the capacity to generate behaviorally relevant sensorimotor transformations, then what is the contribution of the neocortex and hippocampus? Although specific functions of these mammalian circuits were not examined, we can still make some inferences about the role of cortex in these behaviors.

It is possible that the cortex and hippocampus play an important role in allowing the animals to exhibit variable behaviors in response to the same stimulus input. For example, wildtype mice can switch from an escape response to a freezing response even in the presence of an ongoing threat stimuli display. This shift in the behavior of the wildtype mouse has several advantages. Under testing conditions without shelter, the animal does not have a safe place to run. By switching its behavior output from an escape response to a freezing response, the mouse can conserve its energy until the predator physically strikes at which point the mouse has to choose between fight or flight responses or until the threat is gone. Also, if there are multiple predators in the area and there is no safe place to escape, freezing would decrease the likelihood of the animal being detected by the other aerial predators. The acortical mouse, on the other hand, remains reactive and elicits an escape output as long as it detects the threat signal. Neocortex and hippocampus in this case might give the animal a survival advantage by providing flexibility in behavioral output, allowing different circuits of escape or freezing to be activated under the same conditions and preventing repetitive outbursts of activity.

A similar repetitive behavior in acortical mice is observed during the initial phases of exploration in the maze. These mice spend tens of minutes in a quadrant of the maze before they venture out to another quadrant, only to repeat the same steps of exploration within the new subspace of the labyrinth. They explore the same set of end nodes repetitively. This behavior slowly changes over the course of the experiment, but every single acortical animal exhibits the same repetitive exploration of subspace behavior during their initial bouts. It is possible that the cortex and

hippocampus allows for variable exploration strategies in the wildtype mice, and therefore we see a higher efficiency of exploration among the wildtype mice, at least in the early phases of the experiment.

In addition to providing flexibility and variability in behavioral responses in wildtype mice, the neocortex and hippocampus may carry a safety signal that is lacking in the acortical mice. Although, the acortical animals can learn to navigate and find the location of a reward successfully in a complex labyrinth, the same animals never made it to the shelter under threatening conditions. Borrowing some terminology from psychology, we can think of the labyrinth as a 'kind' learning environment. The rules of the labyrinth never change: physical structure, reward location, and access to home cage are never altered. There is no element of surprise. Once the water location is discovered, all animals explore the maze freely and collect water rewards as they please. The predator avoidance assay on the other hand is a 'wicked' learning environment. The animal is not given any prior knowledge about the environment and the conditions can change dramatically in an instant. Therefore the cortex and hippocampus may be providing or storing a safety signal which might be required for directed escapes to shelter in wicked learning environments such as the predator avoidance assay, but not for navigation and goal-learning in kind learning environments such as the labyrinth. These points remain as scientifically guided speculations until further studies are conducted to understand the role of cortical and hippocampal circuits in animal behavior.

5.3 Future Directions

Throughout evolution, the neocortex and hippocampus may have taken over a significant portion of sensory processing, learning, and memory functions in humans. The great expansion of these structures must have allowed us, humans, to have certain cognitive capabilities that clearly separate us significantly even from our closest phylogenetic relatives. Therefore, the neocortex and hippocampus will probably always remain as the most studied brain regions in neuroscience. When we started this project, the original paper characterizing the acortical mice had stated that the animals did not have intact vision. We have shown that, by using naturalistic behaviors, we can assess the capacity of the subcortical circuits. Several studies [42, 43] have already made use of these results and several other projects are ongoing. In fact, we have preliminary results showing forepaw reaching behaviors in acortical mutant mice. Experiments testing social behaviors, sleep cycles, and long-term memory in the mutant mice would help us identify the evolutionary advantage of neocortex in

rodents. Additionally, the results of the structural and functional validation experiments suggest that many other research projects focusing on deep brain structures might benefit from using this structural mouse model to understand what aspects of an observed behavior are governed by the subcortical circuits and what aspects may require the presence of cortical and hippocampal circuits. I see this work as providing support for the significance of subcortical circuits in animal behavior. Focusing on naturally occurring animal behaviors, one could argue that the most striking implications of behavior control reside within the subcortical circuits: the role of arcuate nucleus in hunger [36], the role of subfornical organ in thirst [45, 56], the role of ventromedial hypothalamus in social interactions [44], and the role of superior colliculus in defensive behaviors [17, 42], just to name a few. This is not to conclude that neocortex and hippocampus do not play a role in these behaviors and have a direct effect on the subcortical circuits. However, we should be wary of research that emphasizes models of cortex that fail to integrate the contribution of subcortical circuits and attempts to explain brain function using models that purely rely on cortical pathways and arbitrary stimulus-response paradigms, especially when using non-human model organisms.

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Appendix A

MATERIALS & METHODS

Experimental Animals

Emx1-Cre (Strain 005628) and C57BL6/J (Strain 000664) mice were purchased from Jackson Labs. Pals1 flox/flox mouse line was a gift from Seonhee Kim and Christopher Walsh. Acortical mutants were bred by crossing Pals1 flox/flox mice with Emx1-Cre line. During embryonic development, exon 3 of Pals1 flanked by loxp sites is deleted by Cre recombinase expressed in Emx1+ cells. Breeding pairs with heterozygous acortical mutant progeny and Pals1 flox/flox mice were set to generate the homozygous mutants. Surgical removal of neocortex and hippocampus in C57Bl6/J mice was completed by aspirating tissue through bilateral craniotomy. The void was filled using a low toxicity silicone adhesive (WPI, KWIK-SIL). Males and females aged 12-48 weeks were used for this study. Animal husbandry and experimental procedures involving animal subjects were conducted in compliance with institutional guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) and by the Office of Laboratory Animal Resources at California Institute of Technology.

Histology and Imaging

Animals were anesthesized with ketamine:xylazine. Following transcardial perfusion with PBS (1x) and PFA (4%), tissue was harvested. Cryostat or vibratome coronal sections were collected at 100 μ m. Imaging was completed using Olympus Slide Scanner and confocal microscopes. For MRI imaging, one of each genotype males from the same litter were perfused with PBS with 5mM gadolinium-DTPA (Gd-DTPA) followed by 4% PFA with 5mM Gd-DTPA. The brain was left intact inside the skull and samples were stored at 4°C when not being imaged. All magnetic resonance microscopy was performed using an 11.7 T vertical bore Bruker Avance system equipped with Micro2.5 gradients and a 25 mm internal diameter linear birdcage coil for RF transmission and reception. For imaging, mouse brains within the skull were mounted under 5mM Gd-DTPA in PBS with 0.01% sodium azide within a flat-bottomed glass test tube. Ambient temperature during scanning was maintained at 25°C via the gradient cooling system. High resolution T2*-weighted 3D gradient echo images were acquired with TR/TE = 50/5 ms, flip angle = 35

degrees, matrix = 256 x 256 x 256, field of view = 16 x 16 x 16 mm, voxel size = 62 microns isotropic for a total imaging time of 7 hours 17 minutes per sample. ITK-SNAP and ImageJ were used for analysis.

Predator Avoidance Assay

An animal was placed in an infrared-transmitting black acrylic arena (86cm L x 47cm W x 30cm H) and allowed to explore the arena and shelter (if available) freely for at least 10 minutes. Behavior was recorded using two cameras mounted on the frame as the arena providing side view and bottom view. Prior to any visual stimulus presentation, experimenter monitored the animal ensuring that the animal explored inside the shelter. As the animal approached the center of the arena, visual stimulus was presented in the upper visual field of the animal. Black and white looming disk expanded from 2° to 50° in 500 ms. White receding disk shrank from 50° to 2° in 500 ms. For each trial, one type of stimulus was presented 10 times with 1 sec inter-stimulus-interval (ISI). Visual stimuli were presented using custom code in Psychtoolbox in MATLAB or Unity (Unity code provided by Yang Liu). Following stimulus-offset, the behavior was recorded an additional 5 minutes. Animals were group-housed and were only tested with one type of stimulus in a given day to reduce the likelihood of habituation to the arena. Videos were analyzed using DeepLabCut to extract body positions and the output was analyzed using MATLAB 2017b, 2019a and GraphPad Prism 9 for further analysis.

Maze Navigation and Reward Learning

All acortical (mutant and lesioned) animals were single-housed for the maze experiments. Following 24hr water deprivation, the animals were placed in a home cage attached to the maze. Animals had access to food in their home cage and were free to explore the maze overnight (max 12 hrs). For further details, please see [60].

Appendix B

INVESTIGATING THE RETINOTECTAL PATHWAY IN WILDTYPE MICE

In terms of our understanding of the brain, retina is probably the best example for what is possible with scientific investigation [49]. The connectivity between the retinal ganglion cells and their outputs has been studied extensively [48]. Yet, we still do not have a clear understanding of which retinal ganglion cells send direct projections to which type of neurons in the superior colliculus. We decided to investigate this problem by making use of genetically identified neuronal populations in the superficial layers of SC (sSC) and identify the type of RGCs that project to a specific neuronal population. We used trans-synaptic retrograde labeling pseudotyped rabies virus injections in sSC of mice which had Cre recombinase expressed in a given subset of neurons [7] (Figure B.1, A). To investigate the retinotectal connectivity in the mouse, we mapped monosynaptic RGC inputs to tachykinin-1 (Tac1) expressing neurons, which have been shown to label superficial layers of SC [6].

Approximately 90% of retinal ganglion cells (RGCs) send projections to superficial layers of SC in the mouse [16]. The pseudotyped rabies virus encodes green fluorescent protein which would be expressed only in the RGCs that are presynaptic to the Tac1+ neurons. We found that Tac1+ neurons in mouse sSC receive direct input from the retina (Figure B.1, B). We identified the different RGC populations projection to the Tac1+ neurons using antibody markers and RGC dendritic arborizations. The alpha RGCs were identified using osteopontin antibody staining, the direction selective ganglion cells (DSGCs) were labeled using CART antibody staining in the mouse retina. We analyzed colocalization of the antibody marker with eGFP expression from the rabies virus. We did not find a significant overlap (<1%) with the alpha cells in the whole-mount retina (Figure B.1, B). This result decreases the likelihood that Tac1+ neurons in sSC as the looming detectors downstream of the retina since it is speculated that transient-OFF alpha ganglion cells are responsible for the looming reaction [70].

Unlike the Tac1+ neurons, we found that Rorβ+ neurons in superficial layers of SC do not receive direct input from the retina.

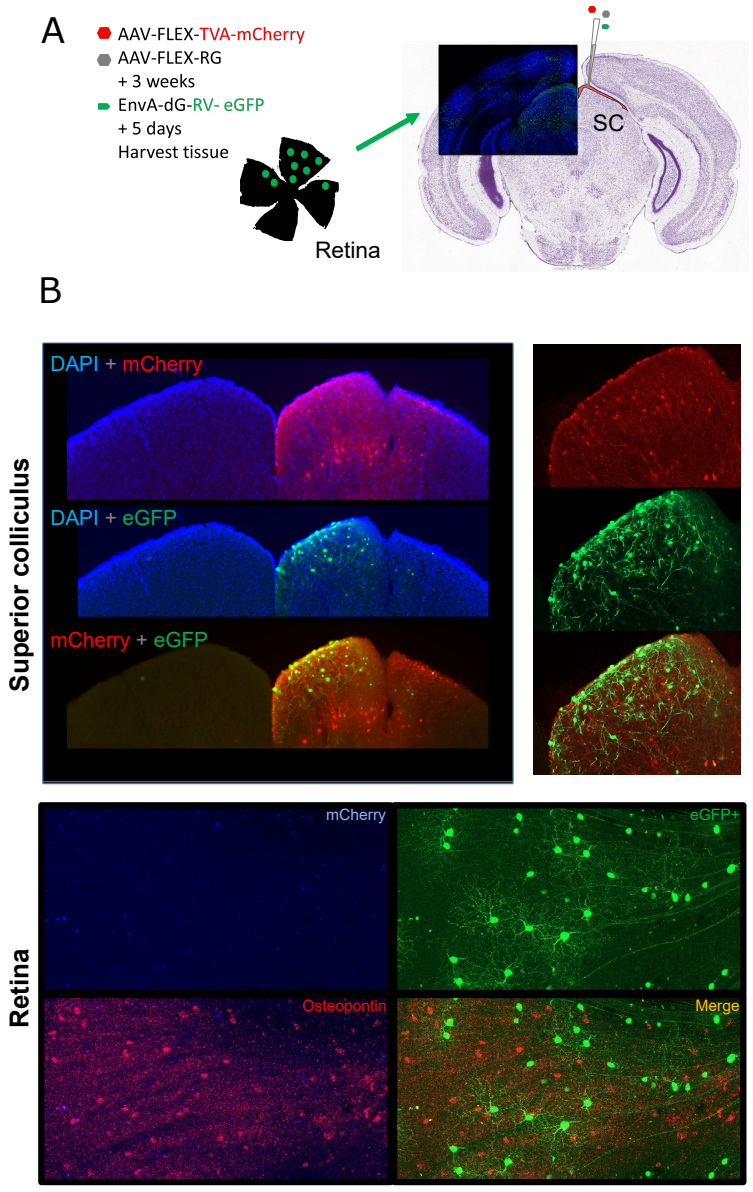

Figure B.1: Retinotectal connectivity analysis via monosynaptic retrograde tracing in mice. (A) Methodology used for monosynaptic retrograde tracing from genetically identified neurons of the superficial SC . (B) Transsynaptic tracing of Tac1+ SC neurons reveal retinal connections that avoid -RGCs: Representative images of viral spread in superficial layers of SC. Local connections within SC (Top). Neurons expressing both mCherry and eGFP are starter cells. Retrogradely labeled RGCs showed <1% overlap with -RGCs (bottom).

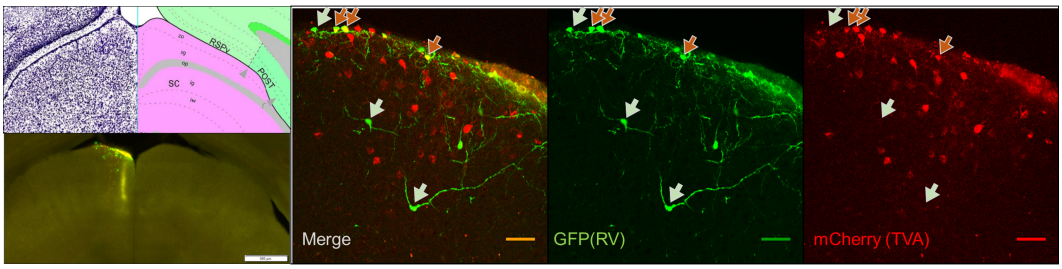

Figure B.2: Rorβ neurons in superficial layers of superior colliculus receive local input, but not from V1. (A) Epifluorescent image of virus injection site in Rorβ-Cre animal. Scale bar, 500 µm. (B) Confocal images of the same section showing Rorβ+ starter cells (red arrows) at the injection site in sSC expressing both the receptor protein TVA and rabies virus encoding GFP. Within the sSC, there are neurons that express only GFP, indicating that they are local presynaptic partners (white arrows) of Rorβ neurons. Scale bar 50µm.

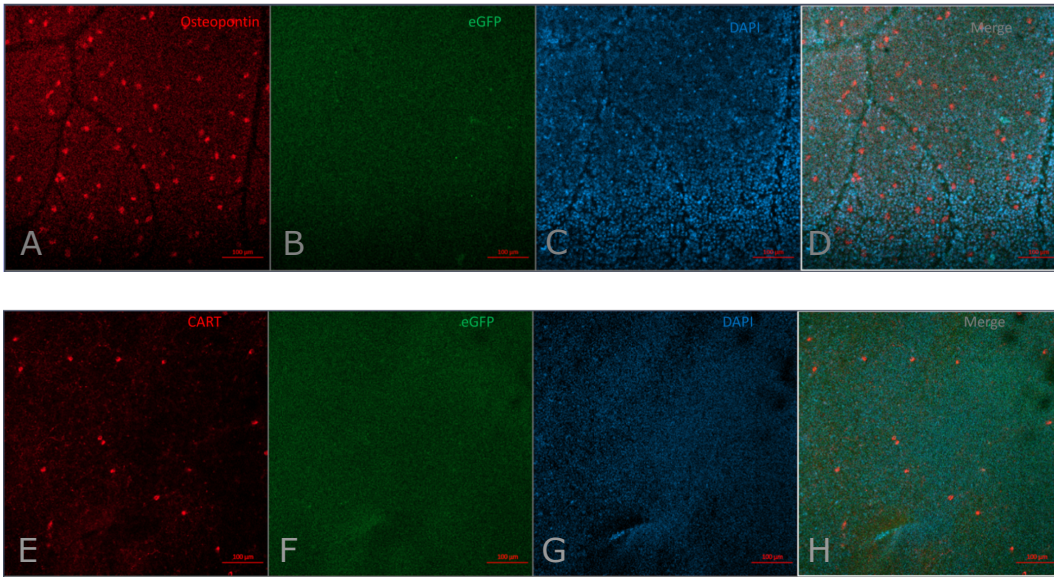

Figure B.3: Rorβ Cre+ neurons in sSC do not receive direct input from RGCs. (A) Whole-mount retina showing stained with osteopontin antibody and (C) DAPI in a Rorβ Cre+ animal injected with pseudotyped rabies virus expressing eGFP. (B) No retrogradely labeled eGFP+ retinal ganglion cells (RGCs) were observed in the retina. (E) Whole-mount retina stained with CART antibody and (G) DAPI. (F) Similarly, no RGCs labeled via retrograde viral tracing in another animal. Merged images for shown in (D) and (H). Scale bar 100µm.

Appendix C

CELL-TYPES WITHIN THE HONEYCOMB STRUCTURES IN THE MOUSE SUPERIOR COLLICULUS

The cholinergic projections terminate within the intermediate and superficial layers of superior colliculus, leading to a modular structure resembling a honeycomb. These cholinergic projections mostly arise from the pedunculopontine tegmental nucleus and their function within the mouse superior colliculus is still unknown [64]. The modular structure of the cholinergic projections to the mouse SC helped us validate the development of the subcortical circuits in the acortical mutants.

Using a previously published molecular map showing expression patterns of calcium binding proteins, synaptic proteins, and transcription factors within the different layers of SC (Figure C.1, A) and other published results [6, 62] as a starting point, we investigated neurons that co-localize, border, or exhibit a pattern along with the cholinergic projections within SC. Calbindin+ neurons show co-localization with these cholinergic projections (Figure C.1, B). Although previous studies were not able to define a specific role for the cholinergic inputs to SC using chronic extracellular recordings, future studies could make use of our results for functional imaging studies. For example, a Calbindin-Cre & CHAT-Flp mouse line can be used to sparsely label SC neurons with Cre-dependent calcium indicators and to optogenetically activate the cholinergic inputs to assess the functional role of the honeycomb structure.

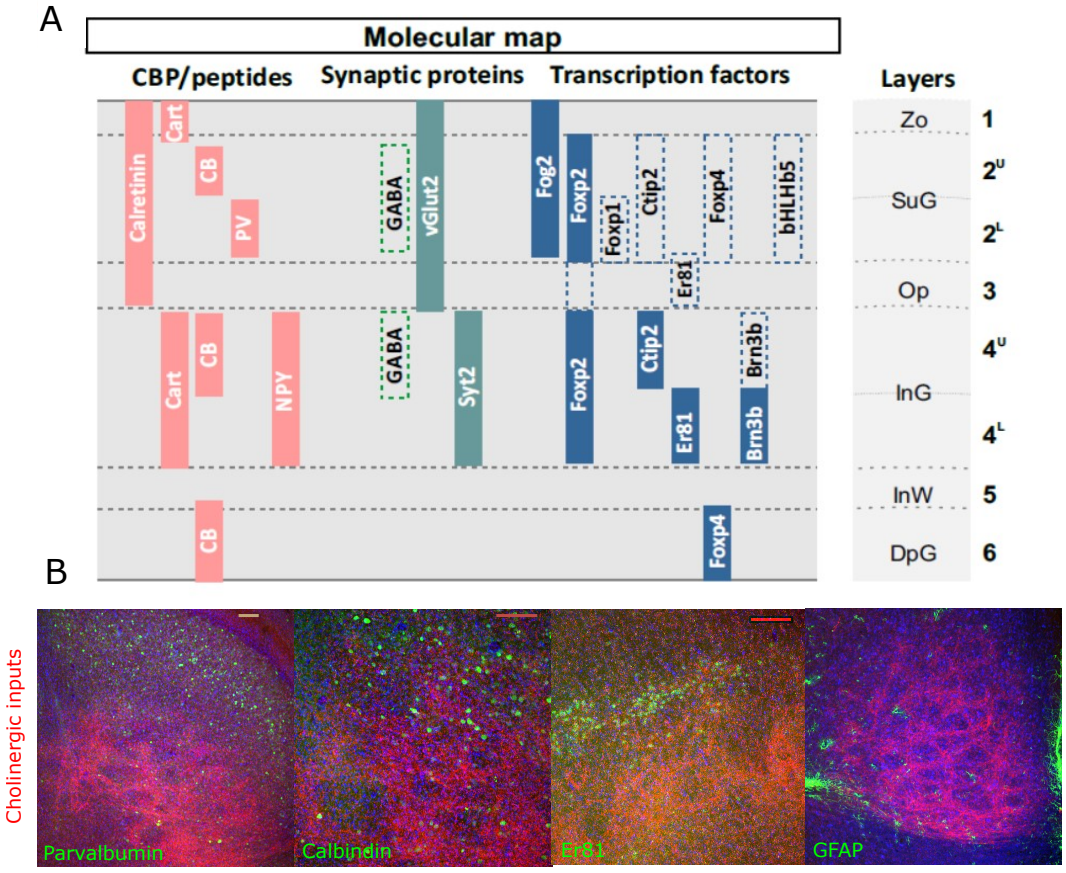

Figure C.1: Cholinergic inputs to superior colliculus. (A) Molecular map within the layers of mouse superior colliculus. Adapted from D.L. Rousso, Society for Neuroscience poster presentation. (B) Horizontal sections stained with vesicular acetylcholine transporter antibody for labeling cholinergic terminals in red, cell body staining in green.

Appendix D

ACTIVITY-DEPENDENT LABELING IN SSC

Immediate-early-gene (IEG) expression is a powerful and reliable tool for identifying groups of neurons following a specific stimulus or behavior [53]. There have been attempts in recent years to find a group of neurons that are activated and labeled with IEG antibodies within the mouse superior colliculus in response to the black looming disk [72]. Unfortunately, this is not an easy task since the IEG expression, in this case c-fos, requires the same neurons to be active for extended periods of time. In the looming assay, the animal is freely moving and with every move of the animal, the location where dark expanding hits the retina changes, leading to different groups of downstream neurons to be activated.

Using a head-fix setup, we were able to guarantee that the visual stimulus remains in the same location in the upper visual field of the animal, allowing theoretically the same groups of neurons to be active downstream of the looming sensitive retinal ganglion cells. In addition, due to the highly controlled nature of the experimental setup, we were able to put a block separating what one eye sees from the other. This simple modification allowed us to have an internal control: one eye can be exposed to one stimulus and the other can be exposed to a control condition. The resulting effect should be observed in the contralateral superficial layers of SC. Given the retinotopic map of the sSC [13], we can predict where the active neurons should be within the sSC based on the visual stimulus location. The looming disk, flashing disk, or background conditions were tested.

Following a headplate surgery, the animal was headfixed on a headbar and was allowed to run freely on a treadmill. Black expanding stimulus (max. size at 20 °) was presented on the upper visual field of the animal for 30 minutes with one second ISI. An hour after the stimulation, the animal was perfused with 4% PFA in PBS. The brain was post-fixed overnight at 4°C. Coronal sections were stained with c-fos antibody.

Given the retinotopic map of the superficial layers of SC, we can predict that the active neurons should be medially located within the sSC and be present only on the hemisphere contralateral to the stimulated eye. As we predicted, we detected a group of c-fos expressing sSC neurons contralateral to the eye which received the

visual stimulus (Figure D.1, A). In contrast, we did not observe any labeling on the other hemisphere.

We tested different stimulus conditions using this head-fix set up: both eyes receiving the black looming stimulus, both eyes receiving only gray background (no stimulus), one eye receiving black looming disk and the other eye receiving black flashing disk (size of flashing disk is the same as the maximum size of the looming disk; 20°), and other controls (Figure D.1, B). Similar patterns of activity were observed in mutant mice indicating that the retinotectal pathway connectivity and function in mutant acortical animals are similar to the wildtype mice.

Another study reported activity-dependent IEG labeling in intermediate layers of SC, but it failed to show a difference in IEG activity in the mouse sSC in response to looming stimulus [72]. Since the retinal ganglion cells project directly to the superficial layers, a causal response is expected to be in the superficial layers of SC.

Our results show a clearly labeled group of neurons in response to the visual threat with an internal control. This experimental set up is an ideal target for tagging neurons in activity dependent manner [24, 61]. Following the permanent labeling of these neurons, future studies can use tracing techniques to find upstream and downstream synaptic partners for elucidating the full circuit for the visually evoked defensive behaviors.

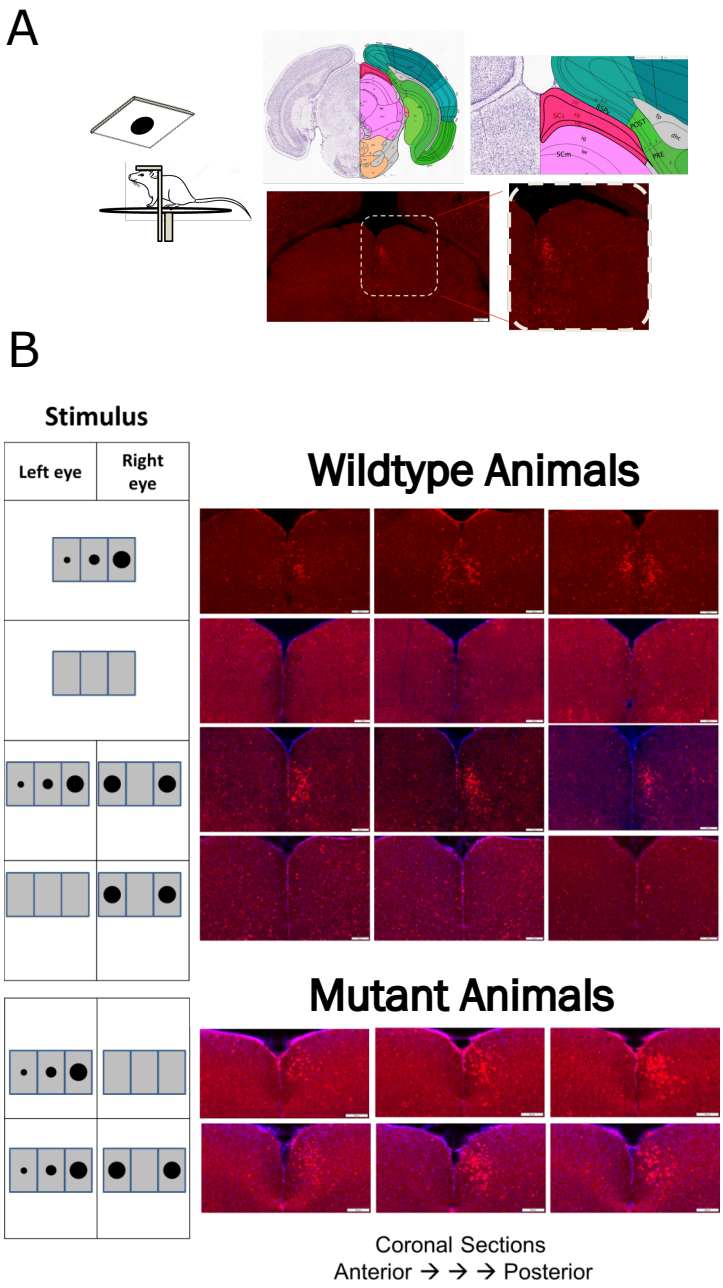

Figure D.1: Activity-dependent labeling in sSC in response to visual stimuli. (A) Head-fix setup with dark expanding disk stimuli presented on a monitor in the upper visual field. Corresponding retinotopic location in sSC shown in coronal section (100um) of medial sSC, contralateral to the eye that was exposed. (B) Stimulus conditions with corresponding coronal images of c-fos expressing neurons in sSC in wildtype and mutant mice.

Appendix E

FEAR-INDUCING ODORS

Mice can detect odors released by their predators in nature. The odor-induced fear responses have been studied in the laboratory setting. 2,3,5-trimethyl-3-thiazoline (TMT) is a chemical found in fox feces that has been shown to induce defensive and avoidance behaviors in rodents [69]. Using C57Bl6/J mice, we tested TMT and 2-propylthietane (2-PT) [1] (Figure E.1, A). A single mouse was placed in a clean cage without any bedding material and the animal was exposed to the predator-odors in a ventilated hood. Similar to other reports [18], we did not observe robust defensive behaviors in response to TMT or 2-PT [1].

A relatively recent study [30] claimed to develop "potent innate-freezing inducers termed thiazoline-related fear odors (tFOs)." This study used "2-methyl-2-thiazoline (2MT), which induces a level of freezing comparable to that induced in the learned condition." The researchers first paired a neutral odor with foot-shocks. Then, the animals were placed in a Y-maze. One arm of the maze led to the learned fear odor and the other arm had 2MT. The results showed a striking 100% avoidance of the 2MT arm (Figure E.1, B).

We tested 2MT with the expectation of observing robust defensive behaviors. Instead, the animals became inactive and unresponsive within minutes. The animals did not respond to touch or loud sounds (Figure E.1, C). We also tried fox urine (https://www.thepeemart.com/) and did not observe any robust behavioral responses in C57BL6/J animals. This might be due to strain differences or laboratory mice losing their sensitivity to certain odors as they are not exposed to natural stimuli in breeding colonies. The research group which introduced 2MT as an innate fear-inducing odor has published more recent articles on the same subject. The behavioral responses of the mice when exposed to 2MT are being characterized as artificial hibernation [52] and hypothermia and anti-hypoxia [51]. Fear-inducing predator odors and the behaviors they evoke in mice are open questions and should be studied further. The acortical mutant mice would be an interesting model to test with the predator odors given the persistent and robust behavioral responses they show when exposed to visual threats.

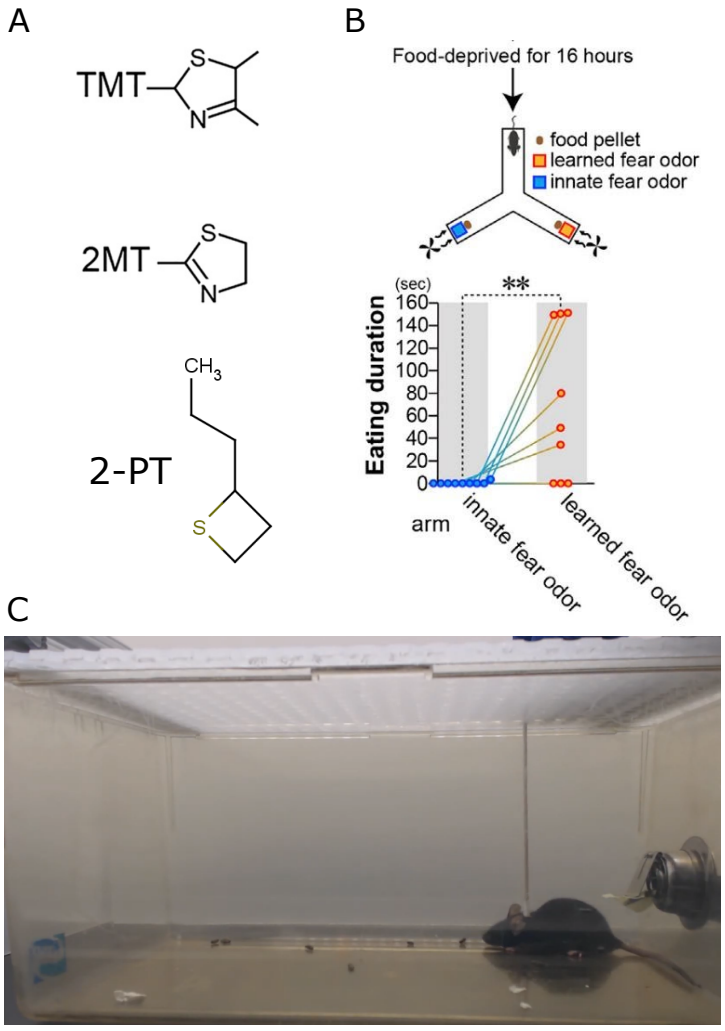

Figure E.1: Fear-inducing Predator Odors. (A) Chemical structures of fear-inducing odors: 2,3,5-trimethyl-3-thiazoline (TMT from fox), 2-methyl-2-thiazoline (2MT), 2-propylthietane (2-PT from weasel). (B) Figure from [30] claiming innate fear behaviors in response to 2MT. (C) Attempt to replicate the results from panel B. C57BL6/J mouse tested with 2MT placed on a filter paper in a ventilated cage being gently poked with a cotton swab. After a few minutes, the animal is unresponsive to sound or touch.

Appendix F

ONE-SHOT LEARNING OF EFFICIENT PREDATION

The predator avoidance task does not require any learning, therefore the innate circuit must be built-in and ready to perform. I found this was not the case for prey capture behavior. In nature, mice hunt insects in addition to foraging for other nutrition sources. Recent studies have shown that mice use both auditory and visual sensory information while hunting crickets, relying mostly on their vision [28]. These studies did not address learning of hunting behavior. Laboratory mice are not exposed to foraging and hunting tasks and therefore are not familiar with insects. In these studies, researchers provided crickets in the home cage of the mice, allowing the animals to overcome any neophobia and have pre-testing opportunity to "hunt" insects. In our experiments, we were interested in investigating the first hunting experience of a naive mouse.

The animals were single-housed and acclimated to the arena for 3 days. Following <24hr food deprivation, the mouse was placed in the arena, this time with Blaptica dubia (roach). On Day 1 of testing, we observed that some wildtype mice learned to hunt the roach very fast, whereas others took hours. We defined time to capture as the initiation of a successful capture with partial or full consumption. The large variance observed for Day 1 dropped drastically on Day 2 and remained as such for the trial Days 3, 4, and 5. The wildtype animals required only one experience before exhibiting efficient hunting behavior indicating that a prey capture is a one-shot learning task. Following a single experience, the animals show persistent and fast hunting behaviors.

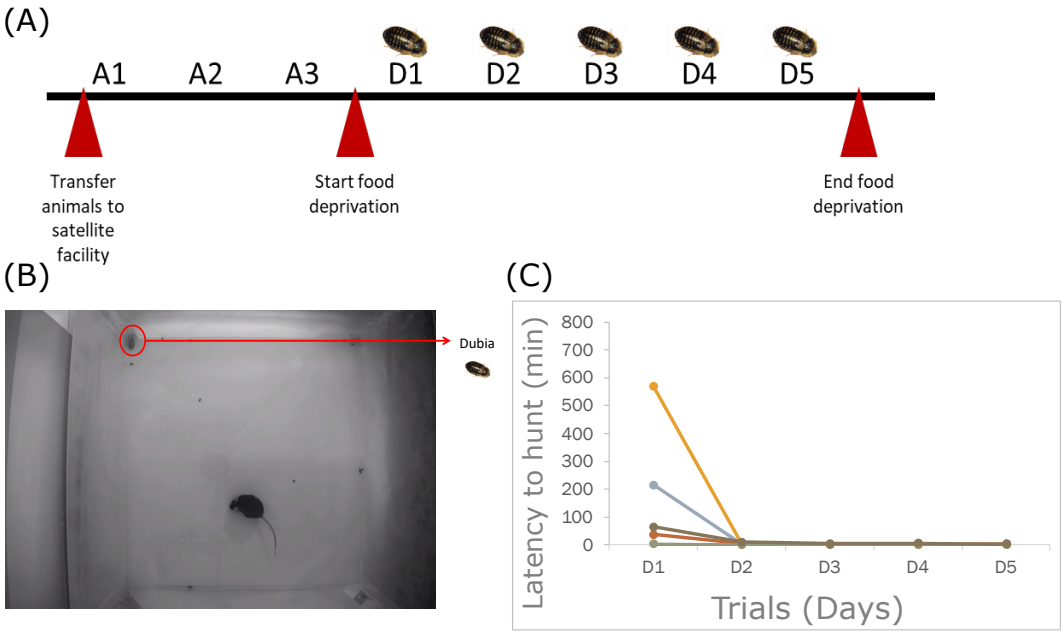

Figure F.1: One-shot learning of efficient hunting in mice. (A) Experimental timeline of prey capture assays. (B) Prey capture arena, mouse, and dubia as seen in recordings. (C) One-shot learning of efficient hunting of insects in laboratory mice indicated by the decreased time to capture after one trial. The increased efficiency in hunting is persistent over the next four days of testing.

Appendix G

TISSUECYTE FOR WILDTYPE AND MUTANT MICE

The projects listed in my thesis have focused on a subset of subcortical regions for the validation assessments. Previously published papers did not provide coronal section images for the whole brain. The images below can be used to confirm the intact subcortical regions in the homozygous mouse (e.g., intact striatum, cerebellum, and inferior colliculus can be seen (Figure G.3). Some hippocampal and neocortical structures remain in the heterozygous mouse (Figure G.2). Heterozygous animals were tested in the predator avoidance assay. Similar to the acortical mutants and lesioned mice, heterozygous acortical mice showed multiple escape bouts in response to black looming stimuli. The heterozygous animals also failed to escape to shelter during the visual threat presentation. Although these animals have some neocortical and hippocampal tissue remaining, their behaviors were not distinguishable from the homozygous mutants.

TissueCyte Images of Wildtype, Heterozygous, and Homozygous littermates (In collaboration with Hongkui Zeng, Allen Institute for Brain Sciences). Corresponding individual images from serial two-photon tomography are available for each brain imaged [71].

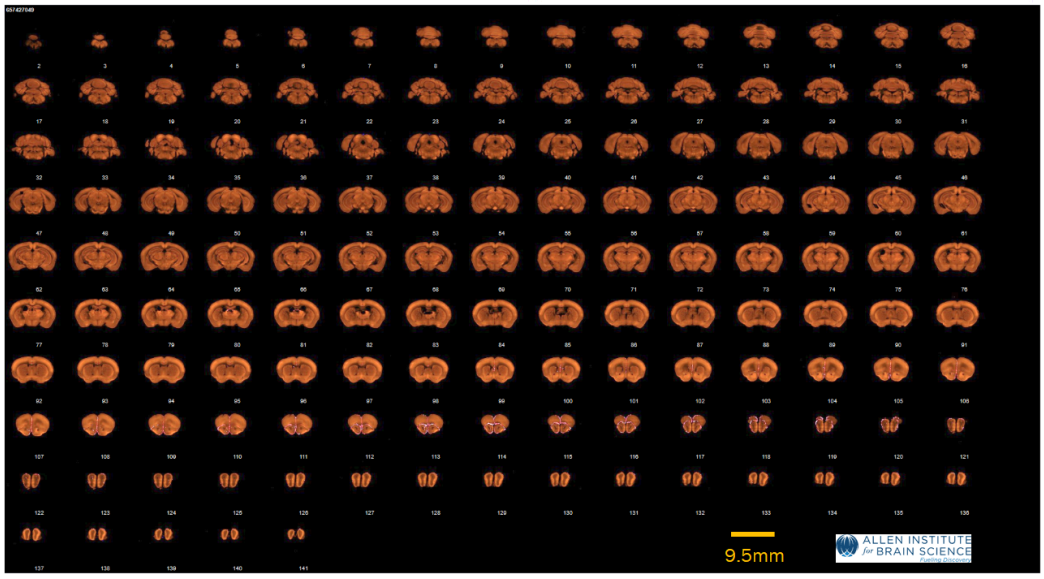

Figure G.1: Wildtype littermate: Pals1+/+: Emx1-Cre+

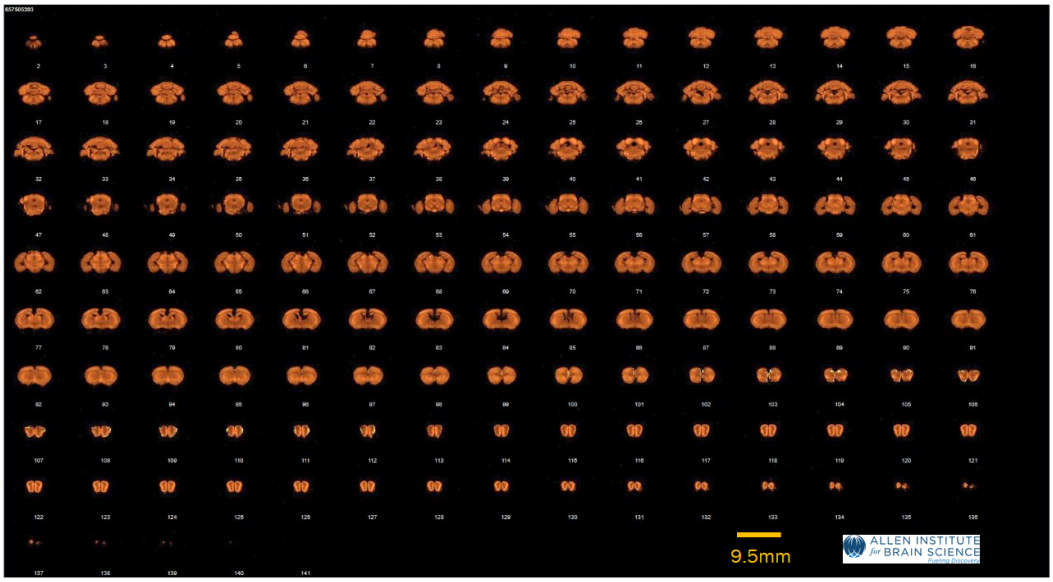

Figure G.2: Heterozygous littermate: Pals1loxp/+: Emx1-Cre+

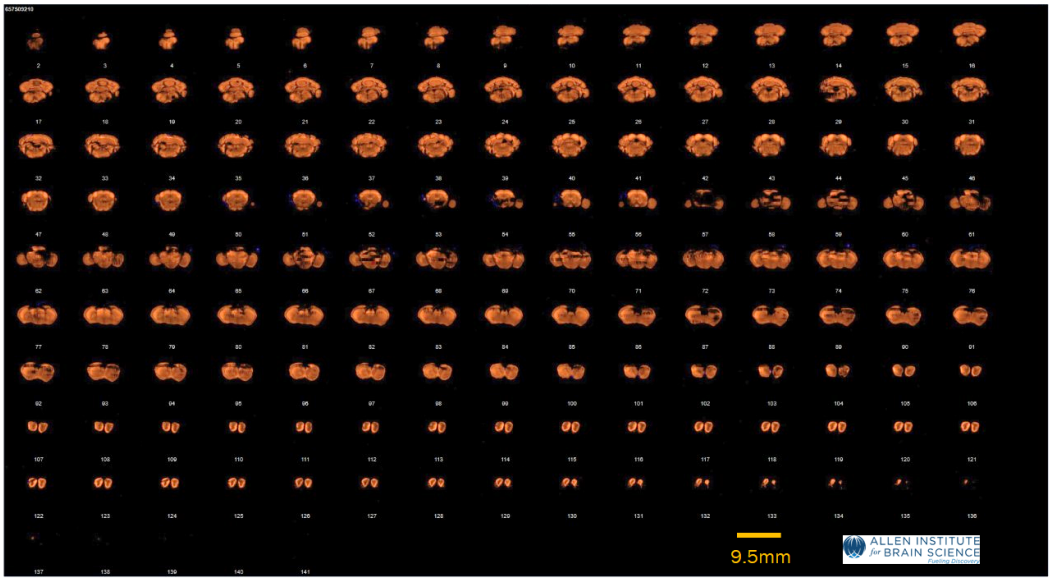

Figure G.3: Homozygous littermate: Pals1loxp/loxp: Emx1-Cre+

Appendix H

NESTING DEFICITS IN MUTANT MICE

Nesting is a natural behavior observed in laboratory animals [12]. All laboratory mice are provided nesting material in their home cage. The wildtype mice shred this material and fashion a compact nest in the corner of the cage (Figure H.1, top panel). Acortical animals fail to make a nest in their home cage; the nesting material remains intact on surface or slightly buried in the bedding (bottom panel). This behavioral deficit is interesting since the mutant animals are not able to exhibit directed escape to shelter in predator avoidance assay. Understanding the neural correlates of nesting behavior can help us answer if the subcortical circuits lack the capacity to form safety-related associations.

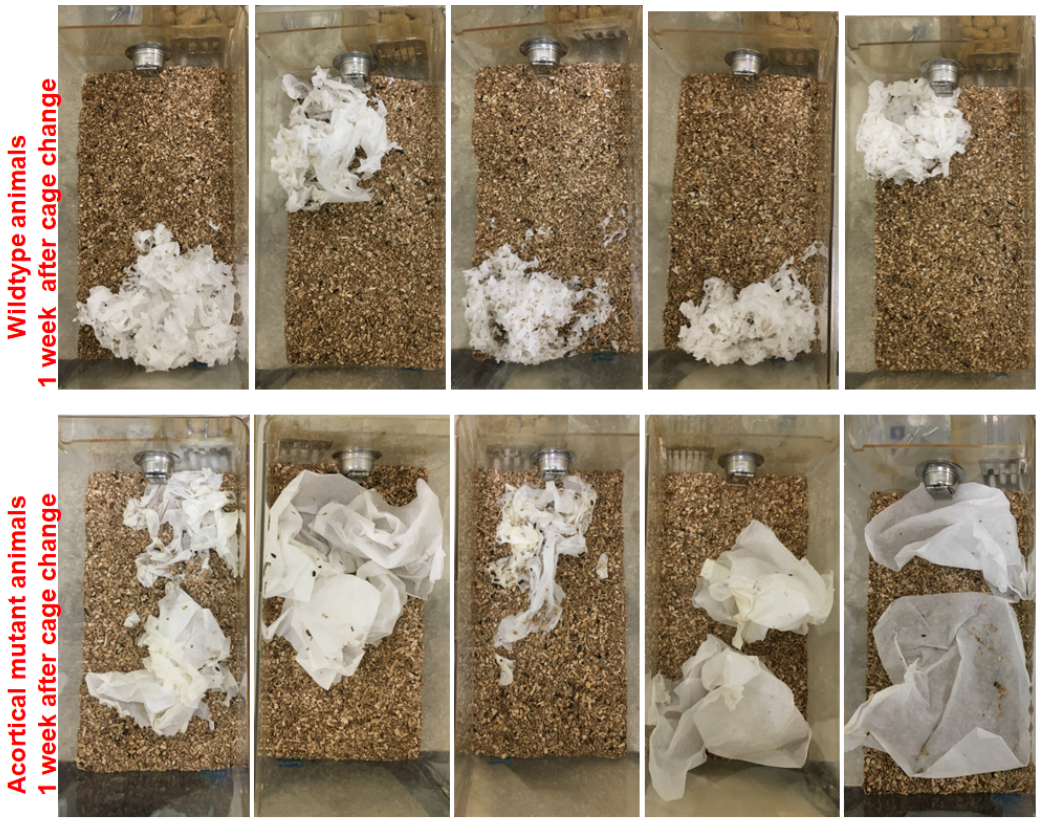

Figure H.1: Nest building in wildtype and mutant mice. Home cages of five wildtype (top) and five mutant (bottom) mice are shown. The images are taken at the time of weekly cage change.

Appendix I

REPRODUCTIVE SUCCESS

Throughout these experiments, generating the homozygous mice has been challenging. The original paper characterizing these mice reported that the homozygous mice had lower birth weights [34] which is considered a proxy for poor health outcomes [57]. These animals also exhibited motor deficits. The homozygous mice had a very short latency to fall from a wire hang test when compared to wildtype and heterozygous mice. There may be other deficits not described previously. For example, due to Emx1 expression in the kidneys [4], these animals might have deficits in other organs leading to poor health outcomes.

Heterozygous mice were able to reproduce and give a mix progeny of wildtype, heterozygous, and homozygous mice. When crossing two parents which are heterozygous for both Emx1-Cre and Pals1-flox genes, 18.75% of the progeny is expected to be homozygous acortical mutant mice. We also used heterozygous male or female for both Emx1-Cre and Pals1-flox genes crossed with a breeder which was homozygous for Pals1-flox. Without the Cre-expression, homozygous Pals1-flox mouse is a normally developed, wildtype mouse. This breeding scheme was expected to yield 25% homozygous acortical mutant mice out of the progeny. Despite the different breeding schemes, the number of homozygous acortical mutants which reached adulthood remained low.

Despite different breeding schemes, we failed to get a viable progeny from a homozygous acortical mutant breeder. Given the low number of homozygous animals, we did not explore the breeding of homozygous mice any further. Instead, the animals that reached adulthood were used for predator avoidance, labyrinth, and other experiments in the lab. Among the homozygous animals that reached adulthood, some were removed from the experiments due to health conditions such as penile prolapse, lesions, and other medical complications. The increased likelihood of adverse health conditions might have affected the homozygous acortical mutants' reproductive success. These are observations from breeding schemes. However, future studies should investigate other naturalistic behaviors of the mutant mice further including mating behaviors.