Quantifying visual attention in the mouse using behavior, electrophysiology, and wide-field calcium imaging

A Thesis

by

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CHAPTER 1: Introduction

Vision plays a main role in how we understand and interact with the world. At any moment, our brains take in large amounts of visual information – more than we can consciously pay attention to. Before we're aware of it, the brain quickly decides what's important and shifts our attention towards those things. However, we still don't understand how the brain's neural circuits make this possible.

Studying neural circuitry in humans remains challenging; luckily, the neural circuitry underlying visual processing is remarkably similar across mammalian species. Mice and humans share basic brain structures, including a common basic cortex organization that has specific areas devoted to each sensory input, like sight and sound (Carandini & Churchland, 2013). The initial steps of the visual processing hierarchy, which is the order of brain areas that process visual information, is also conserved. In a simplified way, visual information moves from the retina to the lateral geniculate nucleus (LGN) to the primary visual cortex (V1) and then on to higher visual areas (HVAs). This means that by studying these neural circuits in detail in animals like mice, we can begin to understand how these circuits might underlie visual attention in humans.

It has been established that activity in the primary visual cortex (V1) affects visual perception in humans, monkeys, and mice (Martínez et al., 1999; McAdams & Reid, 2005; Speed et al., 2019). But we're only beginning to understand how higher regions of visual cortex help us focus our attention and make sense of what we're looking at (Moran & Desimone, 1985; Treue & Maunsell, 1996, Wang & Krauzlis, 2018, You & Mysore, 2020). The goal of this thesis is to explore how the brain controls visual attention by studying this process in mice. These goals will be achieved by standardizing behavioral training in a visual attention task and establishing techniques for validating and measuring neural responses. The neural responses can be measured both electrically and optically, all while mice focus attention to particular locations in space.

Vision researchers can measure visual perception by showing a controlled visual stimulus (like a bar or a pattern) and measuring behavioral responses to these stimuli. By having mice report that they perceive the stimulus with a behavioral response (such as a movement), researchers can measure the process of perception. Training mice to report when they perceive a visual stimulus correlates their behavior to their perception, allowing the researcher to understand what the animal is seeing. When there is a very intense stimulus, the animal is likely to perceive and report it quickly. When the stimulus is faint, the animal might not perceive it, and will miss the trial, or may take longer to recognize that the stimulus has appeared. When working with animals, like mice, researchers train them to respond to certain visual cues, and reward them when they correctly respond. Water is often used as a reward, as water intake can be controlled easily using a water-restriction protocol, and then small drops of water can be dispensed when the animal correctly performs a trial. Because of the prior water restriction, the water reward for reporting the perception of a stimulus is motivating to the mouse and elicits quick and reliable behavior. Simultaneously, researchers can record the neural responses in the brain region of interest and compare the response of the neuron when the animal correct or incorrectly performs the task. This helps to understand how visual perception works.

By making these tasks challenging, by showing a faint and hard-to-detect image, we can explore the limits of what the animal can perceive. Difficult tasks are especially useful for studying attention, because they show how focusing in one area can improve performance. For example, a mouse might miss a faint stimulus in an area it isn't paying attention to but will detect it more often when it is attending to that location.

Attention is the way in which the brain focuses more processing power on something important and ignores what isn't. Attention enhances the response to important stimuli and reduces the response to unimportant stimuli. This helps to prioritize the information entering your senses. There are several types of attention, including attention that focuses on different features (like color, motion, shape), attention that focuses on objects, attention that focuses on timing, and attention that focuses on a specific sensory input. Spatial attention, which focuses on a particular location in space, is commonly used when studying attention because it's easy for researchers to control (using visual stimuli and experimentation areas with controlled lighting) but reflects the same underlying principles as other types of attention, so can be used to learn more about attention broadly.

Tasks like the Posner cueing task (Posner, 1980) are commonly used to study attention in primates. In this task, primates were presented with a visual cue that indicated the likelihood of the next stimulus appearing on the left or right sides (either an arrow in the more likely direction, or a plus sign indicating equal likelihood). They found that reaction times to cued areas were faster than when there was an equal likelihood which was, in turn, faster than when the stimulus appeared in the uncued area.

Monkey studies offer an advantage over human studies because precise, invasive recording techniques can be used, and at the same time much of the brain function is conserved. Complex behavioral tasks in monkeys have proven essential for understanding the neural basis of perception, and it's control by cognitive factors such as attention. Monkeys have been trained to attend to visual stimuli in one area of visual space while ignoring stimuli in an alternative area of visual space (Moran & Desimone, 1985). It has also been shown that the complexity and difficulty of the task play a role evoking attention in monkeys (Lavie & Tsal, 1994; Swallow & Jiang 2013). In monkeys, response latencies and variability decrease with attention (Maunsell, 2015; Cohen & Maunsell, 2007), and attention improves response to low contrast stimuli, which is more difficult (Reynolds et al., 2000).

Mice offer genetic manipulations that monkeys do not, which allows for precise manipulations. However, these visual spatial attention tasks designed for humans and monkeys require adaptation for mice since mice are not able to use neutral instructional cues (such as arrow pointing left or right) to guide their behavior.. Inspired by the approach in non-human primates, several labs have developed tasks for mice that elicit selective visual attention. The visual system is largely conserved between humans, mice, and non-human primates, but mice offer several advantages over primates. Advantages include genetic tools, the ease of using multiple advanced techniques (such as electrophysiology and imaging), as well as faster reproductive cycles that allow for increased sample sizes in studies compared to primates. Many different tasks have been adapted and developed for probing selective visual attention in mice. This includes adaptations of many of the behavioral paradigms commonly used in both monkey and human literature to probe attention, which were altered to be useable in mice (Wang & Krauzlis,

2018; You & Mysore 2020; Speed et al., 2020; Speed et al., 2021; Kanamori et al., 2022; Hu & Dan, 2022; Lehnert et al., 2023).

Even though there have been improvements in how scientists study attention in mice, it's still important to create clear and consistent methods for training them. This helps make sure that results can be repeated in different labs and with different mice. However, training mice the same way every time is difficult, because a mouse's behavior doesn't just depend on the task or what it sees but is also influenced by other factors such as recent performance, reward, or the decisions of the trainer (Crabbe et al., 1999; Busse et al., 2011). Our lab developed and optimized a task to train mice in a visual spatial attention task, generating reliable attention across mice and trainers.

Now that we have reliable behavior in spatial attentional tasks in mice, we can begin to understand how behavior and brain activity are related using cell-type specific recording techniques. These include electrophysiology, a technique that can record single-neuron extracellular spiking activity, and wide-field calcium imaging (WFI), a technique that is able to record cortex-wide neural activity using calcium dynamics as a proxy. Electrophysiology uses a thin recording electrode to record the extracellular voltage changes along its depth, which can then be processed to analyze the spiking activity of individual neurons. Electrophysiology provides a high temporal resolution.

With recent advancements in transgenic mouse lines, WFI has also gained popularity. WFI records the fluorescence of genetically encoded calcium indicators (GECIS) (Chen et al., 2013; Dana et al., 2016). When neurons fire, the voltage-gated calcium channels open, triggering an increase in intracellular calcium. Because of this, calcium dynamics can be used as a proxy for neural activity. Protocols have been written and published to standardize WFI rig set-up and initial data processing (Cuoto et al., 2021; Ren & Komiyama, 2021). Easy identification techniques of different visual mouse brain areas using this imaging technique have been established (Zhuang et al., 2017). However, research remains to improve the use and interpretation of the WFI signal, which can have issues with decreasing fluorescence response over time (photobleaching) and other signal confounds (Cardin et al., 2020). WFI has a large field of view, allowing for simultaneous recordings across the primary visual cortex and higher visual areas, which could eventually lead to understanding of what is happening throughout these parts of the visual processing hierarchy in the exact same trial.

This thesis addresses how mice see and pay attention. First, I establish the method for reliably training mice in a standardized, optimized visual spatial attention task, which lets us collect consistent and reliable neural data while the mice are focusing on specific parts of their visual field. Then, I walk through how I validated that the recording electrodes were placed in the intended brain areas during electrophysiology recordings. Finally, I show data collected using wide-field calcium imaging while the mice were learning and performing the visual attention task.

CHAPTER 2. Results

2.1 Reliable training of mice in a standardized, optimized visual spatial attention task

Our lab has previously developed a head-fixed visual detection task for mice, where selective spatial attention was provoked, eliciting behavioral and neural signatures of spatial

attention (Speed et al., 2020; 2021). In this task, head-fixed, stationary (non-locomoting) water-restricted mice respond to stimuli (Gabor grating, varying contrasts) by licking for a water reward (Figure 1A, top). A trial, meaning a singular presentation of the Gabor grating stimulus, begins with a quiescence window during which mice are trained to withhold licking for a predetermined period (Figure 1A, bottom). The quiescence windows last a random amount of time, typically between 3 to 10 seconds. The Gabor grating appears, and the mouse has one second in which to respond with a lick. A correct lick will result in a water reward, a lack of lick in no water, but also no punishment. Spatial attention was provoked by presenting the stimulus in one of two locations - the central (or binocular) visual field, and peripheral (or monocular) visual field - for a block of several consecutive trials before switching to the opposite location for another block of consecutive trials. In this un-cued switch, behavioral effects of attention can be measured as the mouse reallocates the area of visual space it is paying attention to. Successful training in this task reliably elicits behavioral and neural signatures of attention but requires distinct steps due to its complexity.

2.1.1 Optimization of task

We have now optimized a training protocol for this task so expert and naive trainers achieve repeatable metrics of attentional effects.

We trained mice in distinct phases, where the transition point between phases rested on the achievement of specific quantitative benchmarks to evoke reliable and replicable signatures of attention (Figure 1B).

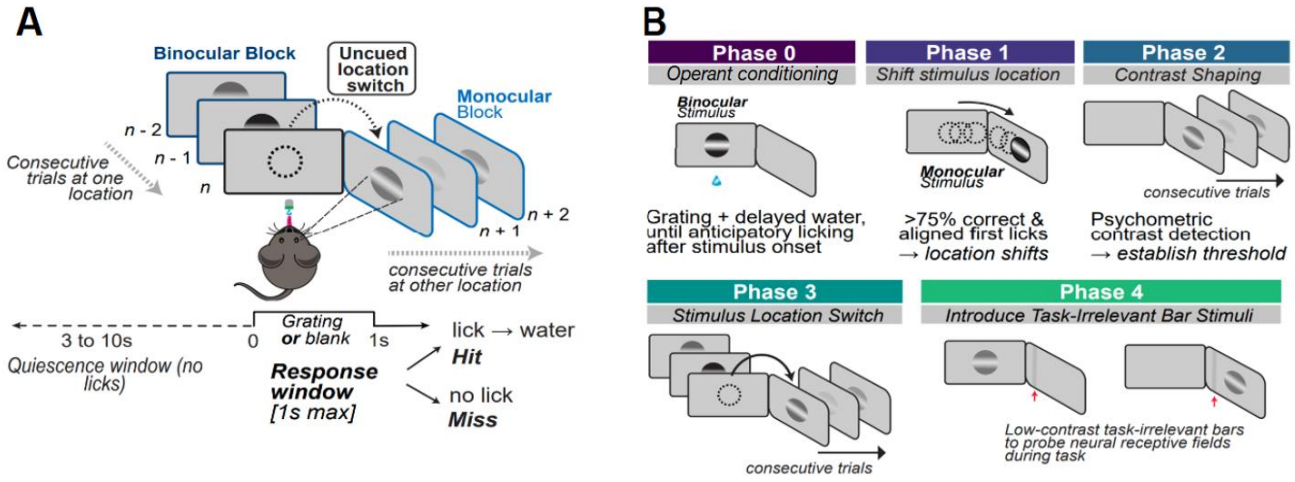

Figure 1: Visual spatial attention task for mice

(A) *Awake, head-fixed mice (N = 25 mice) were placed on a tube and shown stimuli of varying contrasts at two different locations in visual space. If they responded to the stimulus within the 1s response window, they received a water reward.*

(B) *Operationalized phases of training that allow for gradual increasing of task complexity, resulting in more efficient training.*

In phase 0, the mouse first learns through operant conditioning to associate the stimulus with a free water reward and only moves to the next phase when they are demonstrating anticipatory licking after the stimulus onset but before the water reward.

In phase 1, the mouse learns to respond to stimuli in more peripheral regions of space through a slow shifting of the stimulus from the 0-degree location (central; binocular) to the 70-degree (peripheral; monocular) location. Stimuli in the more peripheral regions of space are more difficult to detect (Speed et al., 2019), so 10-degree increments of movement allow the mouse to slowly gain competence. Specifically, average performance drops as the stimulus shifts from the binocular visual field into the monocular visual field but then must recover for the mouse to progress (Figure 2A).

In phase 2, the mouse is exposed to stimuli across a range of contrasts to assess psychometric performance. This allows the trainer to understand whether the mouse's behavior is visually guided, or if a level of luck or disengagement is playing a role. Mice should successfully respond to more than 75% of high-contrast, 'easy' stimuli, and have chance-level performance when detecting faint, low-contrast stimuli. The false alarm rate should be less than 25%. It also serves to establish a "threshold contrast", which is the contrast at which the mouse can detect a stimulus of a given contrast 50% of the time, allowing assessment of improvement across days. The responses to the varying contrasts must be considered psychometric (considering: false alarm rate, hit rate of the easiest contrast, and slope between all contrast responses) for the mouse to continue to the next phase of training (Figure 2B).

The next phase, phase 3, is where the behavioral signatures of spatial attention can first be measured, as it introduces the un-cued switches between locations. In these switches, the mouse must reallocate its attentional resources, which typically takes 5 trials (Speed et al., 2020). In the last phase before neural recording, task-irrelevant bar stimuli are introduced. These task-irrelevant bars are randomly presented throughout the visual field and are stimuli that are known to evoke robust visual responses when presented in a neuron's receptive field. Using these task-irrelevant bars, we can assess neural responses to these stimuli when attention is allocated to specific regions of space. Notably, the task-irrelevant bars do not disturb behavioral performance, but are relevant for analyzing the neural response to non-rewarded stimuli both within and outside of the attended region. Throughout phases 2+, psychometric performance must be maintained to ensure that the mouse is using vision to guide their behavior. When training, the trainer reviews the reaction time and perceptual sensitivity across initial trials after a block switch. Perceptual sensitivity refers to the ability to perceive low-contrast stimuli. Additionally, the trainer reviews the psychometric contrast curve and may change some of the task parameters (such as the contrasts show, quiescence window range of timings, and ratio of blocks in monocular vs. binocular visual space) to make the task more or less difficult to ensure engagement and attention. By optimizing training into distinct and clearly defined phases, mice completed training in an average of 27 days, compared to mice trained without distinct phases who completed training in an average of 72 days, making training 53% faster ($p=0.004$) (Figure 2C). This decreased training time allows for increased output, allowing researchers to train more mice and record more neural data on a shorter timescale.

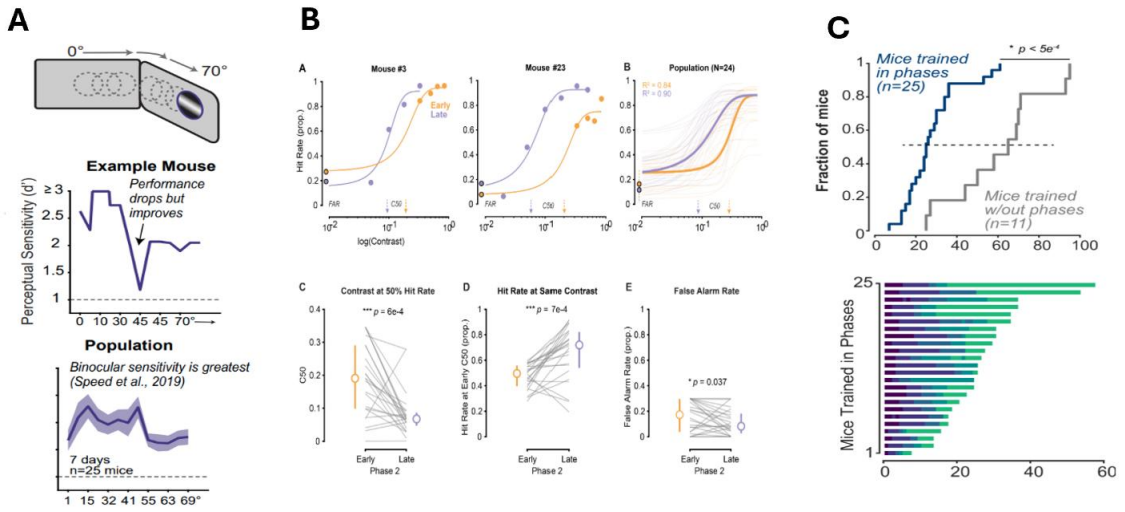

Figure 2: Better behavior through training mice in distinct phases with set benchmarks
(A) *(Top) Progression through phase 1 of training, in which the eccentricity of the shown stimulus shifts from 0° to 70 °. (Middle) shows an example mouse, in which there is a large drop in performance as it shifts to the peripheral visual field, but through training the performance is recovered. (Bottom) shows similar to middle but across the population (N = 25 mice).*
(B) *(Top) Two example mice (a) and population data (N = 24 mice) (b) for improvement in contrast detection throughout Phase 2 training. The threshold contrast (c50, the contrast at which there is a 50% hit rate) was compared in early versus late training (c), as was the hit rate at the initial c50 value (d), and the false alarm rates (e).*
(C) *(Top) Speed (in days) of training mice pre- (n = 11) and post- (n = 25) protocol. Mice trained with the protocol were significantly faster than mice trained without the protocol. (Bottom) Number of days for each mouse trained in the protocol, divided through the use of color to show time spent in different phases of training.*

2.1.2 Behavioral metrics of visual spatial attention

Stimulus location switching reveals behavioral effects consistent with spatial attention. As the stimulus switches from one location to the other, the mouse has both slower and less accurate responses to the stimulus for the very first trials where the stimulus is in this new, unexpected spatial location (Figure 3A-B). When the stimulus continues to appear in the new spatial location the mouse reallocates their attention to that region of space, and both the speed and accuracy of responses improve as a function of trial number after the block switch. Following the protocol, we found that 25/25 mice showed improvements in visually guided behavior consistent with effects of spatial attention.

Next, we wondered how improvements in behavior during visual spatial attention influence psychometric performance. The contrast curves are made by looking at the hit rate across contrasts as well as the rate of random licking. We looked for improvements in the psychometric contrast curve, which is the hit rate across different contrast levels and can show whether behavior is visually guided or not, as well as in the threshold contrast (or contrast at which the mouse has 50% hit rate, also called c50). Both quantities improved across initial trials after a switch in location (Figure 3C), with a shift to the left indicating improved performance.

The analysis provided further proof of the attention effects, because it shows an improvement in perception after spending more time in the new area of visual space. 25/25 mice showed visually guided behavior improved by spatial attention. The key advances for replicability are gradual introduction of complex aspects of the task, and quantitative benchmarks for progress.

Overall, here we establish a standardized behavioral protocol to train mice in a visual spatial attention task and will share these methods to enable other researchers to study the neural basis of visual spatial attention in a rigorous, replicable task. The key advances for replicability are gradual introduction of complex aspects of the task, and quantitative benchmarks for progress. I started as a naïve trainer, and, through specifically following this protocol, successfully trained my first mouse with reliable attentional effects. Naïve trainers with varying levels of previous mouse behavior experience, from undergraduates (including myself) to postdocs have successfully trained mice in the task. . Now, I have trained four mice to show attentional effects. This will allow spatial attention in mice to be studied in a reliable and replicable way across labs, potentially leading to faster developments in our understanding of the neural circuit function that evokes this spatial attention.

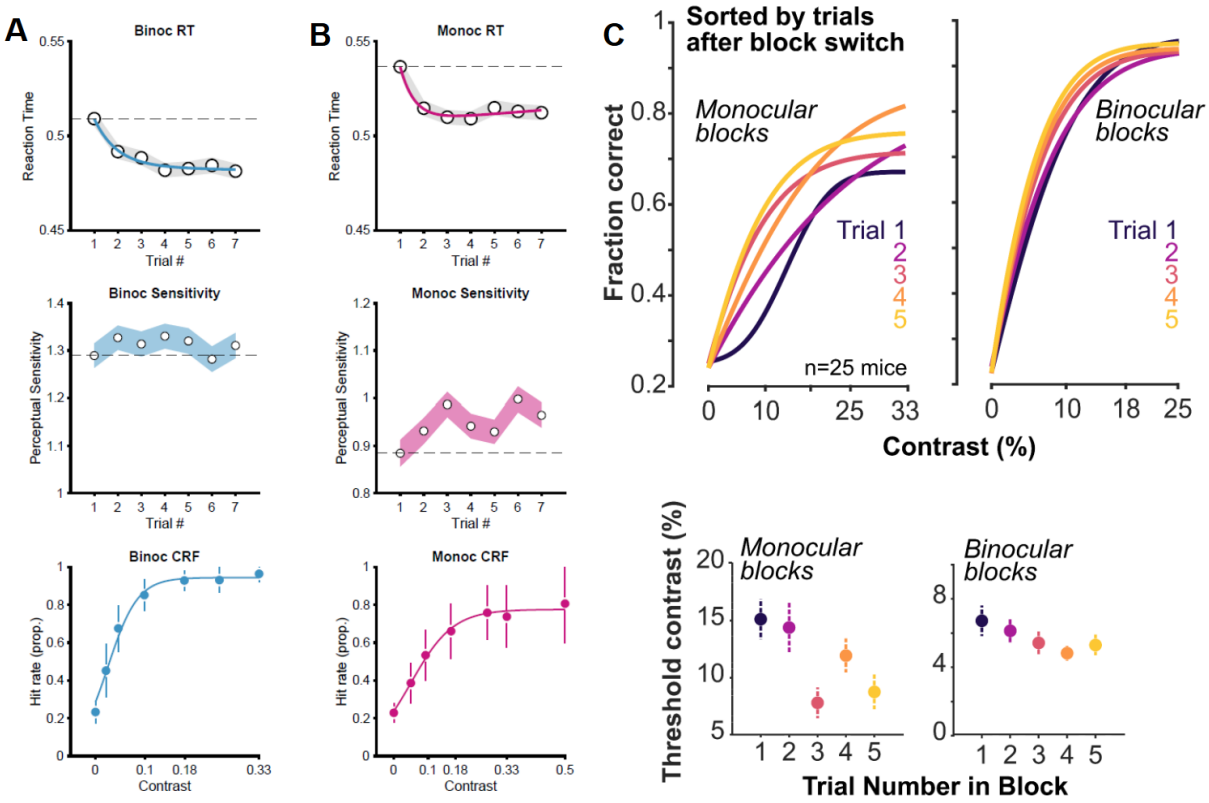

Figure 3: Behavior outputs across all mice trained fully to the protocol

(A) *In the binocular visual field, an improvement in reaction time and perceptual sensitivity is seen across the initial trials in a block in all animals (N = 25 mice) across all days in phases that include switching (N = 537), and the performance remains psychometric.*

(B) *In the monocular visual field, an improvement in reaction time and perceptual sensitivity is seen across the initial trials in a block in all animals (N = 25 mice) across all days in phases that include switching (N = 537), and the performance remains psychometric.*

(C) *Curves showing the hit rate across contrasts, with the random licking rate (response to 0% contrast) included as the bottom of the curve, across the initial five trials in a new location (N = 24 mice, 2051 switches into the binocular location, 1786 switches into the monocular location). Improvement in contrast sensitivity across the initial trials in a block is seen in both the monocular and binocular visual fields.*

2.2 Validation of recording electrode location

A challenge in studying the mouse brain is that it is small. The subcortical brain regions that process visual information are deep within the brain (3mm) and even smaller. Further, their exact positions and shapes can vary across animals. However, since the different areas have different characteristics of their neuronal response to sensory stimuli (i.e. time to respond and amount of information encoded in the response), I hypothesized that these features could be used to classify which area a neural electrode e was recording within. For example, the primary and higher-order visual thalamic nuclei are positioned within 100 micrometers of each other, making it difficult to target recording electrodes (which has a 10mm long shank, so recording result can drastically change based on entry angle) to one thalamic nucleus versus the other. However, the primary visual thalamic nucleus, LGN, and the higher visual thalamic nucleus, LP, are in different positions within the visual processing hierarchy, with LGN responses to the visual stimuli responses occurring earlier than LP. This hierarchical difference creates differences in the first-spike timing and receptive field size that can be used to functionally differentiate between the visual thalamic nuclei (Siegle et al., 2021). With this knowledge of their physiological characteristics, I created a method to determine whether recorded single neurons come from the primary or higher-order nucleus of the visual thalamus. I paired this with histological reconstruction of the recording electrode tracks, which I aligned anatomically with the Common Coordinate Framework developed by the Allen Institute for Brain Science for mouse brain anatomy (Shamash et al., 2018). I combined both the anatomical targeting and physiological characteristics of a single neuron (i.e. latency of first spike, receptive field size) to understand which brain region was targeted during recordings.

Prior to inserting the recording electrode into the brain in awake mice, the back of the electrode is coated with a fluorescent lipophilic dye (DiI) to visualize the trajectory of our neural recordings. The mouse is then transcardially perfused the brain extracted. The DiI probe tracks within 100 uM thick slices of the mouse brain were imaged with a confocal microscope and the acquired images preprocessed for upload to the SHARP-Track software (Figure 4A-B). Preprocessing steps necessary for proper alignment to the Allen CCF (Wang et al., 2020) were completed, resulting in accurate computational alignment of the brain slice and identification of the electrophysiology probe track. To further determine the location using a secondary measure - physiological features of single neurons in the recorded brain region - I coded an algorithm that compares the latency to first spike against the receptive field area, to make an accurate prediction of the location of the recording electrode (Fig 4C). Again, despite the two areas of interest being very close anatomically, they are functionally at different points within the visual processing hierarchy, meaning that neurons in LGN have a shorter response latency and smaller receptive field than LP (Siegle et al., 2021). These two factors were extracted and compared using MATLAB, and the functional location was declared in a figure that also allowed user validation. The combination of analysis on SHARP-Track and MATLAB allows for

additional validations in the classification of the recording electrode location, helping with downstream analysis of neural responses in each location.

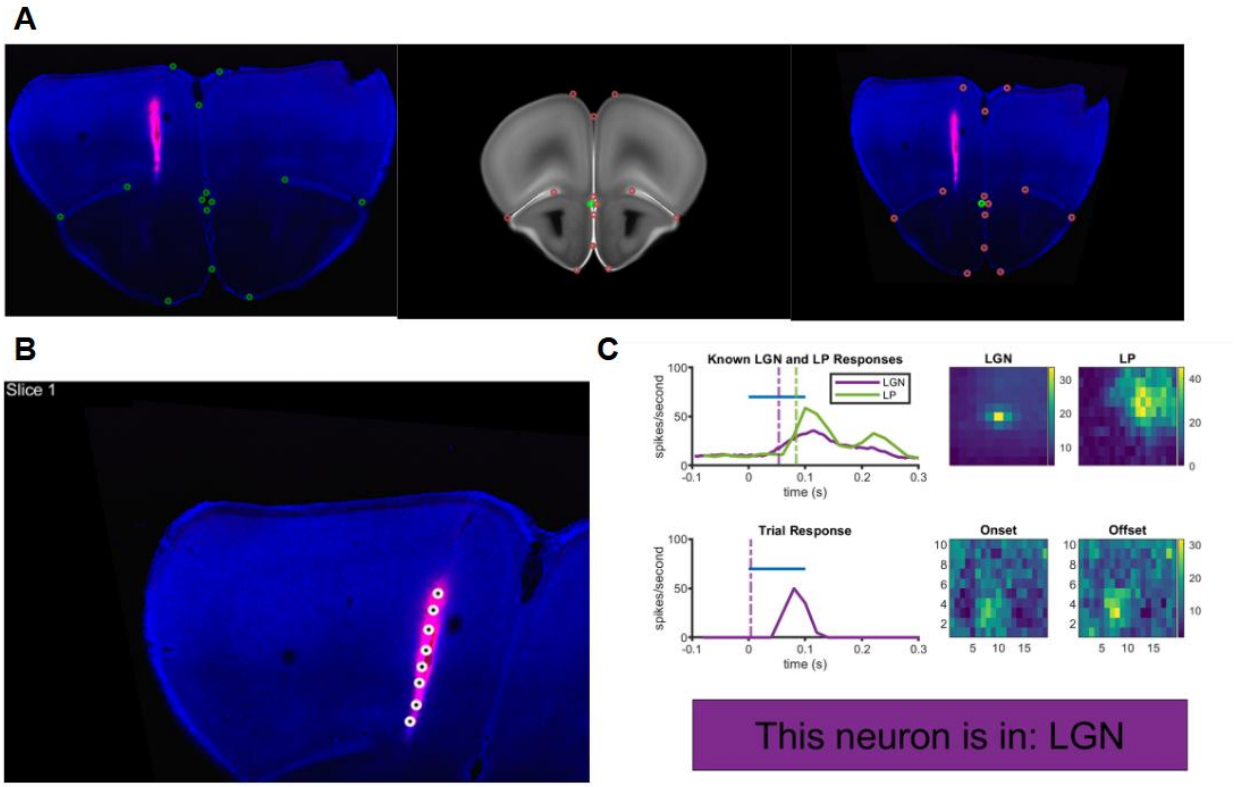

Figure 4: Anatomical and functional validation of probe locations
(A) *Shows the process of transposing a slice onto the Allen CCF, and the resultant aligned image, using an example slice and recording electrode in the anterior lateral motor cortex (ALM). The user first marks key visual features on the experimental slice, before selecting matching visual features from a slice image within the Allen Mouse Brain Atlas.*
(B) *Shows a zoomed-in view of the electrophysiology probe track once the slice has been transposed onto the Allen Mouse Brain Atlas and the probe track identified by the software.*
(C) *Shows resulting figure with functional location result from a recording probe inserted in LGN, where the data from the recording that needs to be localized (middle row) can be visually compared for validation with an example dataset (top row).*

2.3: Visual Cortex-wide Wide-field calcium imaging recordings

To record neural signals, we use both electrophysiology and WFI. As opposed to in vivo electrophysiology, which offers high temporal resolution but low spatial coverage, WFI allows for recording of multiple areas of the cortex simultaneously across days with a large spatial coverage (around 100 mm^2) but lack cellular resolution due to a slower time scale of expression. The large spatial coverage allows us to trace the effects of spatial attention and task learning across multiple visual areas of the brain. Mice were trained in the same visual spatial attention task as described in Chapter 2.1, and the activity throughout the visual cortex was recorded during the task. Through a MATLAB data processing pipeline, the relative fluorescence signals from cortical regions of interest are extracted, aligned to aspects of the

spatial attention task. The outlines of the different regions of interest (which included the primary visual cortex (V1) as well as the higher visual areas (HVAs, specifically: lateromedial (LM), anterolateral (AL), rostrolateral (RL), anteromedial (AM), and posteromedial (PM)) are defined by determining the visual field sign (VFS) map from the calcium responses to stimuli at different locations as in previous studies (Zhuang et al., 2017). A key advantage of WFI is that individual mice can be imaged chronically, allowing data from the same regions of interest (ROIs)to be recorded and analyzed throughout learning of the task

2.3.1 Comparison of hit and miss trial responses

In areas that are involved in the perception and response to the visual stimuli, we expected to see increased neural firing when a stimulus appeared and was responded to. From the training and behavior data, we can analyze the neural responses across the cortex when aligned to trials where the animal correctly detected a stimulus (or 'hit'), failed to detect a stimulus (or 'miss'), and licking when no stimulus is present (or 'false alarm'), as well as the absence of both stimulus and licking (or 'correct reject'). We separated analysis out by both behavioral response and visual area (including V1 and the HVAs), measuring differences in both response timing and response amplitudes that help to better classify the functional hierarchy of the HVAs. In several locations, there is a clear difference between responses to 'hit' (Figure 5A) and 'miss' (Figure 5B) trials (in monocular and binocular V1, LM, PM, AM, the mean peak value across contrasts for hit trials was significantly higher than for miss trials ($p < 0.001$, Wilcoxon signed-rank test)). As expected, within the 'hit' trial responses, there is a clear difference in the neural responses to different contrast gratings, with higher contrast gratings driving increased fluorescence, indicative of increased neural firing.

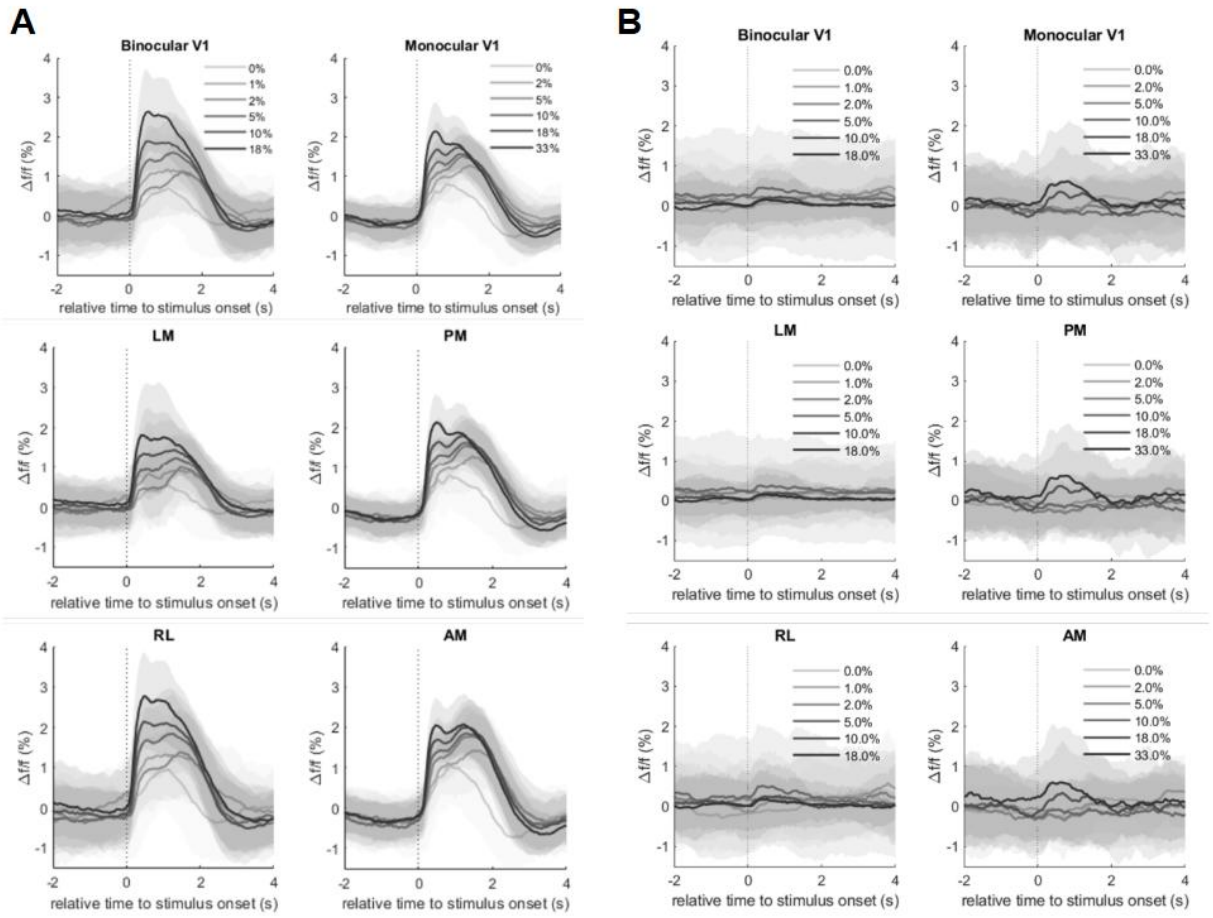

Figure 5: Stimulus aligned responses across the visual cortex

(A) *The fluorescence responses when the mouse correctly reports the presence of the stimulus, or a 'hit'. The coloring of the lines indicates the value of the stimulus shown on that trial, with darker lines indicating a higher contrast stimulus.*

(B) *The fluorescence responses for trials that the mouse did not report the presence of the stimulus, or a 'miss' for matching regions of interest, within the same mice and sessions.*

2.3.2 Extended photobleaching results

WFI is minimally invasive, which allows for chronic recording of the dorsal cortex throughout training. Here, one mouse was imaged daily throughout the learning of the task. A key consideration when performing long-term imaging is the risk of photobleaching. Photobleaching effect commonly occurs during wide-field imaging and refers to the loss of fluorescence of the fluorophores over time due to continuous exposure to the excitation light (Cuoto et al., 2021). Because training must be completed daily for behavioral consistency, it was important to determine whether recording every day of training would lead to significant photobleaching effects, or whether the time between training days (~21 hours) would allow for fluorophore recovery. In analyzing the maximum and minimum delta fluorescence (df/f), the range between the maximum and minimum value grew larger across days, but both did not decrease as would have been expected if there was an extended photobleaching effect (Figure

6). This confirms our ability to record using WFI throughout the entirety of training without decreases in fluorescence.

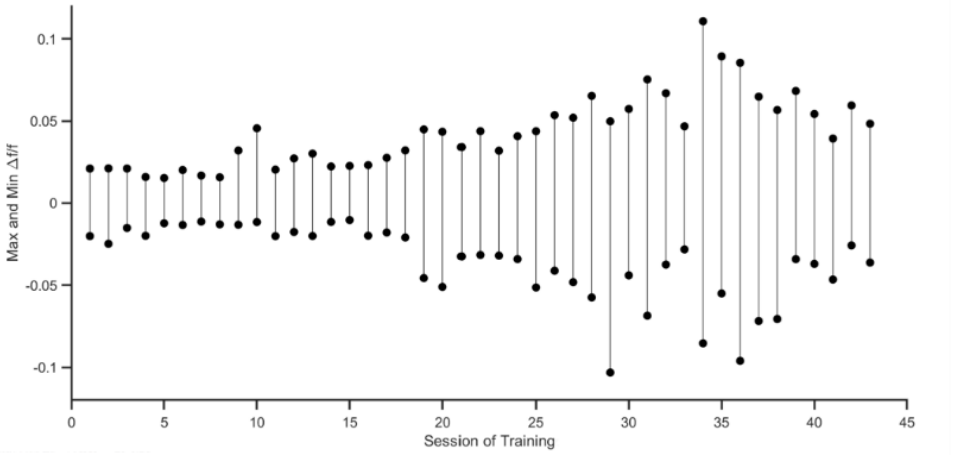

Figure 6: Maximum and minimum delta fluorescence for each day of training (n = 41) in one mouse.

2.3.3 Neural response changes throughout learning of phases

For this pilot mouse, I analyzed the differences in fluorescence responses across training sessions in the different phases of the task, expecting to see changes in the neural responses in V1 as learning progressed. On each day of training, mice perform 3-10 behavioral sessions consisting of 50-100 trials each. I extracted relative fluorescence during training sessions from each distinct training phase to see if there were changes in the neural responses correlated to the learning of the task. There is no statistical power, as the analysis focuses on just one mouse, but the maximum fluorescence trends toward increasing across sessions in phase 0, and the latency of the maximum fluorescence from the stimulus onset trends toward decreasing (Figure 7A). These trends can clearly be seen in the average session-by-session fluorescence responses to stimuli (Figure 7B). In phase 1, the location of the stimulus is slowly shifted from the binocular visual field to the monocular visual field. I mapped the location within V1 that had the maximum fluorescence response during training at each eccentricity location. As would be expected, the location of the maximum fluorescence response shifts posterior and medial as the stimulus location changes azimuths into the monocular field of vision (Figure 7C). In phase 2, for most repeated contrasts, the maximum fluorescence increases as sessions progress. This is exemplified in the 33% contrasts, which were seen in all three sessions, and for which the response grew stronger to (Figure 7D). In phase 3, it trends towards the maximum change in fluorescence increasing across sessions, and the time to the maximum fluorescence decreases (Figure 7E), a result like that seen in phase 0 (Figure 7A). In phase 4, there are similar results to phase 3. Once again, the maximum fluorescence change appears to increase across trials, and the time to the maximum fluorescence appears to decrease, and not just due to changes in contrast (Figure 7F). This might suggest that as the mouse becomes more comfortable with the task, it requires less mental activity to complete. These preliminary data suggest that there are differences in how V1 responds to task-relevant stimuli after learning a part of the task.

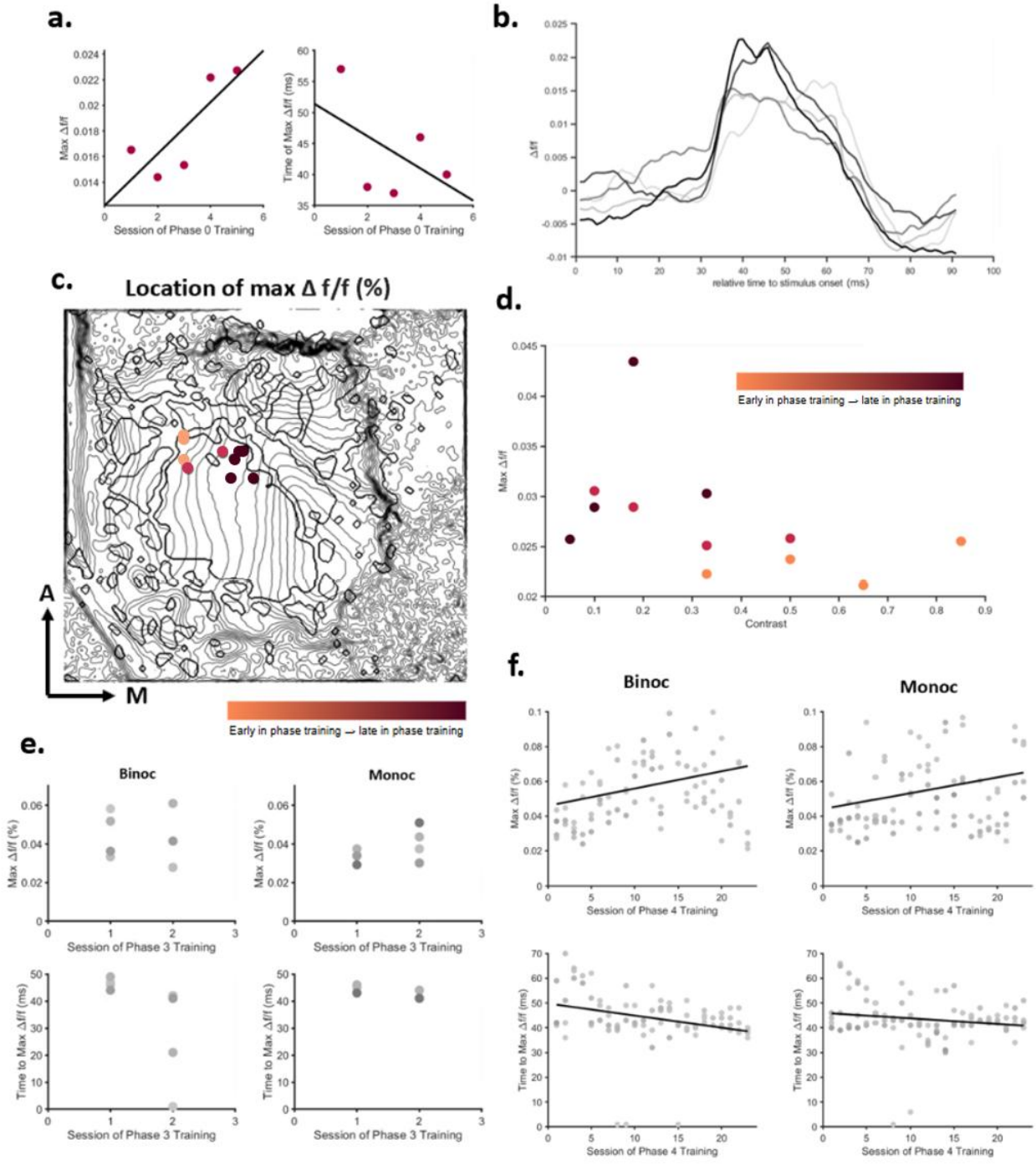

Figure 7: Preliminary analysis of differences within responses throughout training phases.

(A) *Shows the maximum fluorescence response within phase 0 (n = 2 days, 5 sessions).*
(B) *Shows the average fluorescence peak to each stimulus for each session of phase 0, with the coloring of the lines indicating the session number in the phase, where darker colors are*

closer to the end of the phase.
***(C)** Shows the location of the maximum change in fluorescence across sessions in phase 1 (N = 3 days, 10 sessions). The data is overlaid on a contour map of the mouse's visual cortex, with the contours defining responses to different eccentricities of stimuli. Each dot represents a session, and the dot's coloring represents its number into the phase, where a darker color indicates a session closer to the end of training in phase 1. The response moves from binocular areas of V1 towards monocular areas of V1 as the stimulus moves from binocular areas to monocular areas with training.*
***(D)** Shows the maximum change in fluorescence (%) at each contrast for each session in phase 2 (n = 1 day, 3 sessions), with the shading of the dots getting darker to represent the session further into the phase. The maximum fluorescence appears, preliminarily, to be increasing across sessions even at the same contrast.*
***(E)** Shows the maximum change in fluorescence at each spatial location in phase 3 (n = 1 day, 2 sessions) as well as the time to the maximum fluorescence at each spatial location for both sessions. Each dot represents the response to an individual contrast level. Data from additional mice (and sessions) would be necessary to make any conclusions about the effects of training in phase 3..*
***(F)** Shows the same parameters as those shown in E but for phase 4 (n = 7 days, 23 sessions). It appears that the responses are getting faster with increased training, and that the maximum fluorescence response is also increasing across training.*

CHAPTER 3. Discussion

Overall, this thesis has presented a process for reliably training mice in a standardized, optimized visual spatial attention task, which then allows for reproducible neural data to be recorded and analyzed. Additionally, it presented methods for validating this neural analysis when recording using electrophysiology, as well as initial analysis of differences in neural responses across V1 and the HVAs when recording using wide field calcium imaging. Together, these results can be used in future studies to increase understanding of how visual attention is regulated in the brain.

3.1 Replicable animal training

There are limitations in how reproducible animal behavior can be, driven by variations in animal learning speed, motivation, and temperament. From my observations, different animals can have different approaches to the task, and different levels of stamina in completing it. This creates variability in the rate of learning and the success on any given day but is controlled for by having quantitative benchmarks for the transition to learning the next phase of the task.

Non-task factors can also have a large impact on mouse performance, including external noise, any sort of stressors that might occur directly in advance of the daily training, new smells, training at a different time of day than typical, or training in a different location. Some of these factors can be controlled (i.e. training at the same time of day is fairly easy to accommodate with advanced scheduling), but others are difficult to mitigate, especially in a shared laboratory setting (i.e. external noise).

While both novice and expert trainers were able to train mice that had behavioral markers indicative of visual spatial attention, there are also differences in trainer styles and

behaviors that can lead to differences in training. Different trainers take different approaches to troubleshooting similar errors, may accept different levels of variability in their training schedules, or may implement interventions to improve mouse behavior with different levels of urgency. Again, some facets of this can be easily controlled (i.e. continuity of trainer), while others are more difficult to standardize. However, the standardized protocol will limit this variation, thanks to the quantitative benchmarks for moving on to the next phase of training that are listed.

In the future, it may be possible to have semi-automated training. A first step could be to have trainers observe in real-time from a remote location using a live stream of the mouse, which would remove some of the variability in noise and smells, as well as the possibility that the mouse is picking up on cues from the trainer for when to respond. A second step could be implementing an algorithm that can automatically run the next session of training without human intervention, based on training data from past mice and quantitative benchmarks outlined here. This could be trained using the existing training decisions (such as changes in the quiescence length and ratio of trials in each area of visual space) compared to the behavioral outcomes. Occasional human intervention, such as to verify behavior ahead of the transition to a new phase of training, could be included. A model such as this may face difficulties in correctly understanding and interpreting the range of mouse behaviors, and the meanings behind them. For example, we have seen that there could be several possible reasons for a mouse to stop responding entirely, which could mean that the mouse is positioned poorly, the mouse is not adequately motivated, the mouse has completed the day of training and is sated, the task is too difficult, or the mouse is stressed. These instances where similar behavioral issues can result from varying causes could create difficulties in automating a response.

The success of our task in eliciting visual spatial attention by both novice and expert trainers will allow for this protocol to be implemented by other labs, where they can quickly and efficiently train mice to have visual spatial attention. Sharing this protocol across labs will increase the robustness of the results and help to limit reproducibility issues that can arise when studying difficult behavioral paradigms.

3.2 Post-hoc classification of subcortical brain regions in the visual thalamus

The small size of the mouse brain and its many areas can make it difficult to target a specific region when recording using recording electrodes. The use of widely available histology tools in combination with powerful, freely available tools including the Allen Mouse Brain Atlas and accompanying SHARP-Track program allow for anatomical validation that is visualized in an easily understandable way. The ability to combine functional markers with anatomical validations allows scientists to be more confident in the results they are drawing about different brain regions, and readers believe that the targeted areas are correct.

Validation tools like the Allen institute's SHARP-Track, when used in combination with known functional parameters about the area, will make it easier to confidently investigate signals in deeper brain regions, including subcortical regions and regions involved in other sensory systems.

3.3 Understanding the WFI Signal

Wide field calcium imaging offers the advantage of being able to record at a large spatial coverage, but the time scale is slower and resolution lower than electrophysiology. This relatively slow temporal resolution creates some difficulties in interpreting the signal, especially when analyzing the responses to a task that changes relatively quickly and requires a movement to indicate detection. The temporal resolution of wide-field calcium imaging is relatively slow because calcium dynamics are slower and play out over a longer timeline than neural voltage changes (Ma et al., 2016; Ren & Komiyama, 2021). Because of the slow timescale, the motion to prepare for or receive the reward can cause signals that overpower the visual signal, and need to be regressed out (Salkoff et al., 2020; Musall et al., 2019).

One way to address these confounds is to implement an algorithm that can account for the impact of licking and spontaneous mouse motion in the calcium signal that is seen for a 'hit' and 'false alarm' response and may confuse the signal that is just due to visual stimuli. Especially in the higher visual areas, where there is a higher latency to response than V1, it is important to understand the role this motor action is having in the output response, and what the responses look like without it. Another key analysis would be understanding the effect of the reward to see if it is contaminating the visual signal. This has been done preliminarily by comparing rewarded (hit) and unrewarded (false alarm) trials in which a lick occurred, which would both have similar confounds from the motor action, but analysis after regressing out the mouse's movement may provide additional insights.

Additionally, errors may be introduced to the analysis through the current technique of manual selection of regions of interest. This manual selection introduces human error into following the perceived bounds of any of the areas and creates the potential for replicability errors if different people select regions differently. To reduce this potential error, a next step could be creating a code to automatically identify the bounds of V1 and HVAs using the visual field sign map and should not be too technically difficult to implement. Creation of this code would also save significant computational time for the researcher, who would no longer need to complete multiple steps, each lasting several minutes, for the initial processing of each dataset.

3.3.1 WFI signal to analyze learning throughout the task

The initial analysis of changes in neural responses in V1 throughout learning in one mouse leaves many questions that could be improved and answered through increasing the sample size and ensuring that trends are consistent across mice. Once sample size is increased, it would be interesting to extend the analysis to the higher visual areas, to see if any play a particular role in the progression through any phase of the task, or the presence of spatial attention. A possible hypothesis could be that all higher visual areas also see differences in neural responses (including the latency to spike and the maximum response) between the beginning and end of learning a phase of the task, with higher visual areas that are further into the processing hierarchy having more control over learning.

CHAPTER 4. Conclusion

In conclusion, this thesis presents techniques that will allow for increased reproducibility in the study of spatial attention in mice. For the completion of this thesis, I trained six mice, one with imaging and five for electrophysiology recordings in LP and LGN. I compiled behavioral

metrics across all mice trained in the spatial attention task, creating a framework to analyze the behavioral data. I then created codes to analyze much of the behavioral outputs as well. I created protocols for the use of SHARP-Track for slice alignments, as well as histology more broadly. I helped with the completion of the wide-field calcium imaging processing pipeline, specifically helping to extend the codes to process information in the HVAs as well. Through an optimized, rigorous training protocol, combination of functional and anatomical validation of recording locations, and in-depth area specific analysis of results, the neural basis of visual attention can be analyzed with greater replicability and efficiency. This work adds to the current body of research on the subject by increasing understanding of the circuit mechanisms that drive the presence of attention. This will increase the ability to study behavioral and neural aspects of attention.

Materials & Methods

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mouse C57BL/6J	Jackson Laboratory	IMSR_JAX:000664
Mouse Ai32 x Scnn1a-Cre	Jackson Laboratory	IMSR_JAX:009613
Mouse B6.129S4-Gt(ROSA)26Sortm1Sor/J	Jackson Laboratory	IMSR_JAX:003474
Mouse CaMK2-tTA x tetO-GCaMP6s	Jackson Laboratory	IMSR_JAX:024115
Software and algorithms		
MATLAB 2018b or later	The Mathworks, Inc.	https://www.mathworks.com/products/matlab.html
SHARP Track	The Allen Institute	https://github.com/cortex-lab/allenCCF
Allen Mouse Brain Atlas	The Allen Institute	https://mouse.brain-map.org/static/atlas
Hardware and equipment		
Macroscope in tandem-lens wide-field imaging rig	Cuoto et al., 2021	https://doi.org/10.1038/s41596-021-00527-z

Experimental subjects

Male and female C57BL/6J or Ai32 x Scnn1a-Cre or CaMK2-tTA x tetO-GCaMP6s mice were implanted with a stainless steel headplate, which was then covered in KwikCast. This allowed

head-fixation, immobilizing the head and keeping the eyes in fixed locations relative to the presented stimuli displayed on the computer monitors. In some mice, extracellular neural activity was recorded using a Neuropixels recording electrode, for which a headplate with a 3mm diameter recording chamber was implanted. In others, calcium dynamics was measured as a correlate to neural activity, and the skull was made fully transparent using biocompatible polymers that allowed fluorescent light from neural activity to be imaged (Couto et al., 2021). Mice began on a water restriction protocol 24-48 hours prior to training in the task, to ensure motivation.

Recording rigs

Recording rigs were set up with a monitor screen in the central visual field (binocular) and a monitor screen in the peripheral visual field (monocular). A camera in the top right corner recorded the right pupil dynamics of the mouse. The right eye of the mice is recorded using a camera around 22 cm from the animal, using 30 Hz video collection. The mouse was positioned on a half-tube, with a water-delivery spout in front of it, which delivered water in drops to the mouse. The headplate was used to head fix the mouse into the recording rig, so that the central and peripheral visual fields remain the same across days within a mouse. A lick detector, with a beam that is triggered when the mouse's tongue crosses it to receive water, is placed in front of the mouse's mouth and connected to an oscilloscope, so that the precise timing of licking is recorded.

Habituation and training in task

The mice were handled and habituated to the recording rigs for several days before experiments began. Mice were trained to complete a visual spatial attention task through a series of training phases that gradually built in complexity. The mice were first passively trained, where they were automatically given water when the visual stimulus (a Gabor grating) was shown at 0 degrees. Next, the mice were trained actively, so that licking in response to the visual stimulus was required within a short time frame to receive water. The stimulus was then slowly shifted into the monocular (peripheral) field of view in 10–20-degree increments, until arriving at 70 degrees. Next, the stimulus began switching between the monocular (peripheral) and binocular (central) visual field every 10-20 trials, without cue. The introduction of 'switching' was where the beginning attentional signals could be seen, because the mouse reallocated it's visual spatial attention after the switch after the stimulus appeared in the new location for several trials.

Data analysis

Behavioral and neural data was analyzed using MATLAB. Data from each behavioral session was compiled into one table, for easy access across experimental subjects. Eye pupil data from selected sessions was analyzed using DeepLabCut (Mathis et al., 2018). Sessions with less than 20 trials were excluded from attentional analysis, and training days were classified into 6 phases to analyze within.

Histology

Lipophilic dye (DiI, Invitrogen) was applied to the back side of the recording electrode and inserted into the target area. At the end of the recording, mice were perfused, and the brain was stored in PBS before being sectioned into 100 um thickness using a Vibratome. Brains were stained with DAPI (2 mM in PBS, AppliChem), and mounted on slides with a fluorescent mounting medium (Vectashield, Vector Labs), before being imaged using a Confocal microscope (Zeiss).

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