THE PRODUCTION OF INTERVAL TIMING ACTIVITY IN THE PRIMARY VISUAL CORTEX OF MICE

by

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Abstract

The brain is tasked with creating representations of the external world. Often, these representations must include learned associations between a stimulus and what that stimulus predicts. Although historically thought to be the role of “higher-order cognitive” brain areas, it has become increasingly clear that primary sensory areas play an important role in learning and generating such representations. One example is reward timing activity observed in the primary visual cortex (V1) of rodents. In this activity, neurons represent the time interval between a visual stimulus and an upcoming water reward in one of three forms. Both computational and biological experiments have deepened our understanding of this activity, but there remain several open questions related to V1 reward timing activity specifically, and, more generally, to interval timing activity within V1. Here, I provide results that deepen our understanding of how V1 produces reward timing activity by providing a thorough characterization of this activity within the head-fixed mouse and by describing how optogenetically identified circuit elements produce (and aid in the production of) reward timing activity. Together, these results provide evidence that V1 produces reward timing activity in a manner consistent with a theorized network architecture and puts forth novel predictions related to the network architecture underlying reward timing activity. Moreover, I show that this timing activity can correlate, on a trial-by-trial basis, with an animal’s visually-guided behavior when the task demands an animal time an action off of a visual stimulus. Additionally, I show that pseudo-conditioning (i.e., repeated presentations of unpaired visual stimuli), neutral conditioning (i.e., pairing visual stimuli with a delayed visual stimulus), and aversive conditioning (i.e., pairing visual stimuli with a delayed tail shock) result in persistent activity, but that these conditioning strategies fail to generate interval timing activity. In total, the work described here is the result of an interplay between biological experiments and computational simulations that, together,
deepen our understanding of how a neural network represents a time interval. More generally, such an understanding furthers our knowledge of the brain’s capabilities and may allow us to understand fundamental principles underlying complex representations within the brain.

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Chapter 1: General Introduction

It is unclear how the brain creates representations of the external world. Particularly puzzling is the manner by which such responses are produced when these responses relate to the non-sensory aspects of sensory stimuli (e.g., what the stimulus is predictive of in the future). Over the years, “higher-order” areas of the brain have been implicated in producing responses as it relates to an upcoming outcome (e.g., Inagaki et al., 2019) or abstract rules of a task (e.g., Wallis et al., 2001). However, recently, primary sensory areas have been shown to undergo changes when stimuli gain meaning through associative learning (McGann, 2015). Though classically regarded as low-level sensory filters, it has been revealed that these areas play a more pivotal role in producing and aiding in the production of representations of the external world. In this chapter, I will provide an overview of such representations within primary sensory cortex (focusing on the primary visual cortex, V1), how these networks may learn such representations, and how, once learned, these representations are produced within a local network. As it relates to this last point, I will argue that with recent technological advances, we can (and should) investigate these and related questions using a mixture of experimental and computational neuroscience. This process allows each branch of neuroscience research build off of and complement the other. In succeeding chapters, I will show how such a strategy can allow for a greater understanding of how the primary visual cortex produces reward timing activity. By understanding how primary sensory areas produce such responses, we can uncover foundational principles of how the brain creates representations of the external world.
1.1: Primary Sensory Areas Produce Behaviorally-Relevant Representations of the External World

Primary sensory cortex is classically regarded as a low-level feature detector providing simple representations for higher-order areas. In the visual system, representations in early areas relate to simple features, and through the cortical hierarchy, these signals are transformed into complex representations of the external world (Hubel and Wiesel, 1959, 1965; Felleman and Van Essen, 1991). Similar, relatively basic coding strategies have been reported in other sensory areas such as the columnar somatotopy within primary somatosensory cortex (Mountcastle, 1957), tonotopy within the primary auditory cortex (Clopton et al., 1974), and ensemble coding within glomeruli of the olfactory bulb (Buck, 1996).

However, such responses were recorded within untrained (and often anesthetized) animals. These findings do not reflect the potential ability for these areas to have altered responses following associative learning (i.e., instances in which an agent learns that one stimulus predicts another). Typically, associative learning is discussed as the process of learning that a neutral stimulus (called the Conditioned Stimulus, CS, such as the ringing of a bell) is predictive of a salient outcome (called the Unconditioned Stimulus, US, such as food). Through this pairing, the CS becomes more relevant to the agent’s survival and contains more information related to the external world. Although seminal recordings within primary sensory areas do not address how these areas respond following such learning, it has been well established that there is ongoing plasticity within these networks reflecting the learned association (McGann, 2015). Specifically, studies across a range of areas have shown that the strength of neural responses to the CS is modulated following learning. Such findings have been seen within the auditory cortex (Polley et al., 2004; Rutkowski and Weinberger, 2005; Guo et al., 2019), main olfactory bulb (Kass et al.,
2013), primary gustatory cortex (Vincis and Fontanini, 2016), and the primary visual cortex (Makino and Komiyama, 2015; Goltstein et al., 2018).

Although altered strengths have been noted, some instances of associative learning require more complex learning. For instance, when animals, within a virtual reality maze, are trained to discriminate the corridors in which they are running, neurons in primary visual cortex discriminate task-relevant stimuli to allow for appropriate behavior (Poort et al., 2015). Additionally, when animals are trained to run at a certain speed to avoid an upcoming electric shock following visual stimulation, neurons within L2/3 of primary visual cortex acquire a ramp-like activity pattern that corresponds to the expected time of electric shock (Makino and Komiyama, 2015). These studies provide evidence that plasticity within these areas is not confined to simply modulating the response strength and may yet have more complicated alterations to ongoing activity. However, what is not clear is how much of these responses would persist if the CS was removed; in both referenced studies above, the CS co-terminated with the US presentation. As such, it is unclear what neural responses would be like in a window following the CS but prior to the expected time of the US (when there is no external stimulation). Such a question can be addressed using “trace conditioning” strategies.

In instances of “trace conditioning”, the CS and US are separated by a trace delay (typically on the order of ones of seconds). In this trace delay, an agent receives no sensory stimulation between the CS and US and can create an internal representation of the time between the CS and US. Similar to the studies described above, primary sensory areas undergo plasticity following this conditioning strategy. However, in addition to having altered responses, these responses can also reflect the learned temporal interval. For example, when animals learn that a visual stimulus is predictive of an upcoming water
reward after a trace delay, neurons within the primary visual cortex reflect this learned association in a phenomenon referred to as V1 reward timing activity (Hussain Shuler, 2016).

1.2: Reward Timing in Primary Visual Cortex: Learning and Production

V1 reward timing activity was first described in the freely-moving rat (Shuler and Bear, 2006), and it refers to the ability of the primary visual cortex to produce a representation of time between a visual stimulus and an upcoming water reward. This activity is known to take three canonical forms: (1) a sustained increase (SI) or (2) sustained decrease (SD) of activity until the time of expected reward, or (3) a peak (PK) of activity around the time of the expected reward (Shuler and Bear, 2006; Chubykin et al., 2013; Liu et al., 2015). In addition to observing single-unit evidence of reward timing activity, theta oscillations within V1 of trained rats convey the expected time of reward and the experienced reward rate (Zold and Hussain Shuler, 2015); this pattern of activity can be contrasted with pre-training theta oscillations where the duration of cue-evoked oscillations reflect the intensity of the cue itself. Prior studies advance V1 as a substrate in the learning of this timing activity and implicate acetylcholine released from the basal forebrain as a reinforcing signal: lesions of cholinergic axons within V1 block the ability for V1 to learn reward timing activity (Chubykin et al., 2013), pairing visual stimuli with local activation of cholinergic fibers in V1 mimics behaviorally-conditioned reward timing (Liu et al., 2015), V1 timing activity correlates with timing behavior, and perturbation of this activity lawfully shifts timing behavior (Namboodiri et al., 2015; Levy et al., 2017).

To better understand this phenomenology, much work has been done to investigate how V1, as a network, learns to produce reward timing activity and, once learned, how it
produces reward timing activity in its various forms. To address this first question (how does V1 learn reward timing activity), researchers investigated potential learning rules via computational modeling. In this work, it was found that a simulated, recurrent network of excitatory cells could learn timing activity when a visual stimulus was paired with a reinforcement signal (Gavornik et al., 2009). Specifically, the learning rule proposed that within the synapses there is a historical record of what previously triggered spikes (so-called “proto-weights” that act in the same manner as synaptic tags (Redondo and Morris, 2011)). These proto-weights decay over a prolonged period where, upon receipt of a reinforcement signal, they are consolidated into real synaptic weight changes. By iteratively repeating this consolidation process, these synaptic weights are built into the network such that network activity will eventually subtend the interval between a cue and the reinforcement signal. This learning rule, named Reward-Dependent Expression of Synaptic Plasticity (or RDE), proposed a mechanism by which any network of excitatory cells could learn to produce timing activity between a visual stimulus and a reinforcement signal; it is schematized in Figure 1A. With RDE in hand, researchers then sought to uncover what this reinforcement signal could be and focused on the basal forebrain cholinergic system (Chubykin et al., 2013). We have previously reviewed the logic behind studying the basal forebrain cholinergic system (Monk and Hussain Shuler, 2019) and will briefly recapitulate it here.

While the basal forebrain cholinergic system has been implicated in mechanisms underlying associative-learning changes within sensory cortex, few studies provide a thorough understanding of how changes are imparted to a cortical network (Hussain Shuler, 2016). Although the majority of cells within its constituent subregions are not cholinergic, the collection of nuclei composing the basal forebrain (BF) is the major source of acetylcholine (ACh) for cortex (Mesulam et al., 1983). Seminal recordings within the
nucleus basalis of Meynert (NBM, a BF nucleus with the most cholinergic corticopetal neurons (Mesulam et al., 1983)) demonstrate that neurons in this structure strongly respond to salient outcomes, reinforced movements preceding these outcomes, and are able to influence neuronal excitability for prolonged periods of time (Richardson and DeLong, 1991). Such results gave rise to several hypotheses related to the role of ACh in learning. To address the ability of the cholinergic system to act as a reinforcement signal, authors developed a clever experimental design (Chubykin et al., 2013).

Briefly, freely-moving rats were trained that one monocular visual stimulus (Cue 1) predicted a reward at a short delay and another monocular visual stimulus (Cue 2) predicted a reward at a long delay. Once the animals learned this contingency, the rules were reversed where now Cue 1 predicted a reward at a long delay and Cue 2 predicted a reward at a short delay. Prior to this reversal, however, V1 was injected either with saline (control) or with 192-IgG-saporin (experimental). 192-IgG-saporin is a neurotoxin which selectively lesions cholinergic fibers and by injecting it into V1 directly, researchers were able to ablate the vast majority of these fibers within V1 around the recording sites. In theory, within an intact V1, such reversal would result in neurons updating their reward timing activity such that neurons responsive to Cue 1 would update from representing a short delay would now represent the long delay (and vice versa for neurons responsive to Cue 2). This is indeed the case for control animals. However, neurons recorded in the absence of V1 cholinergic innervation maintained, but did not update, their reward timing activity. These results are presented in Figure 1B and show that ACh is a necessary reinforcement signal to learn reward timing activity as its removal precluded the network’s ability to update reward timing activity, but did not influence the ongoing ability of the network to produce reward timing ability. To address the sufficiency of ACh as a
reinforcement signal to engender timing activity within V1, researchers took two independent strategies in vitro (Chubykin et al., 2013) and in vivo (Liu et al., 2015).

Figure 1 Acetylcholine is a necessary and sufficient reinforcement signal for V1 Reward Timing Activity. (A) Top: Schematic of computational model proposed by Gavornik et al., 2009 wherein a recurrent network of excitatory cells (E) learns interval timing when a stimulus (Stim) is paired with a reinforcement signal (R). Bottom: Stimulus-evoked network activity before (gray) and after (black) many pairings. Gray, vertical bar indicates stimulation epoch and dashed vertical line indicates end of the delay window. (B) Left: Task schematic as published in Chubykin et al., 2013. Animals were trained on one contingency (Cue 1 = reward after short delay, Cue 2 = reward after long delay) after which either Saline (yellow) or 192-IgG-saporin (red) was infused, bilaterally into V1. After infusion, the contingency was switched (Cue 1 = reward after long delay, Cue 2 = reward after short delay). Right: Update index score of neurons following reversal learning for saline-infused animals (yellow) and 192-IgG-saporin-infused animals (red) indicate that lesioning cholinergic innervation of precludes the network’s ability to update reward timing activity while sparing its ability to produce reward timing activity. (C) Left: Recording schematic for slice experiments described and presented in Chubykin et al., 2013. L4 pyramidal cells were recorded as white matter (WM) stimulation was paired, at a delay, with carbachol (CCh) application. Right: Neurons recorded in this experiment are able to produce interval timing activity that spans the delay window, in vitro. Prior to CCh pairings (black), activity decayed earlier than after pairing (green). The muscarinic acetylcholine receptor antagonist atropine blocked this effect. (D) Left: Recording schematic as presented and published in Liu et al., 2015 wherein monocular visual stimuli were paired with activation of BF or cholinergic axons within V1. Right: Three example neurons expressing interval timing activity with the SI (left), SD (middle), or PK (right) forms that spans the interval between the visual stimulus (green bar) and time of laser stimulation (blue dashed lines).
Within the same work that addressed the necessity of ACh in V1 to learn reward timing activity, researchers began to address its sufficiency. Briefly, slices of mouse V1 were made and L4 pyramidal cells were recorded from. Recorded neurons were activated via stimulation of the white matter tract (simulating an incoming visual stimulus) and following the recording of baseline evoked responses, these stimulations were then paired (at a 1s delay) with activation of acetylcholine receptors via application of the cholinergic agonist, carbachol. Following this pairing, recorded neurons did, indeed, express long-latency responses to stimulations that extended the length of the conditioned interval (Figure 1C). Researchers then showed that ACh acted as a reinforcement signal via activation of muscarinic ACh receptors (mAChR) through bath application of the mAChR blocker, atropine. Thus, in vitro, ACh is a sufficient reinforcement signal to engender timing activity within V1. However, these results leave open two major questions: (1) can ACh act as a reinforcement signal in vivo and (2) within the intact brain, can ACh promote the production of timing responses in the three canonical forms? To address these questions, researchers then turned to the use of optogenetics and transgenic mice (Liu et al., 2015).

Upon stimulation with blue light, the light-activated cation channel, channelrhodopsin-2 (ChR2) depolarizes neurons and increases their activity (Boyden et al., 2005). ChR2 can be expressed in neurons either via viral-mediated gene transfer (specifically through viral injections within the brain) or selective breeding of mouse lines (to express ChR2 either throughout the brain or in specific subpopulations of neurons). Using these techniques, researchers were able to address whether basal forebrain (BF) or cholinergic input into V1 was sufficient to engender cued-interval activity akin to V1 reward timing activity (Liu et al., 2015). Researchers implanted recording electrodes coupled to optic fibers into V1 of mice that either expressed ChR2 in basal forebrain neurons (mediated via viral-mediated gene transfer) or in cholinergic cells (mediated via selective breeding of
transgenic mice). These animals then underwent conditioning wherein visual stimuli were paired with optogenetic activation of ChR2 in the absence of a reward. In so doing, it was found that either BF or cholinergic activation specifically in V1 is sufficient to produce timing activity in the three canonical, reward timing forms (Liu et al., 2015). These results are presented in Figure 1C and build upon the previously-described in vitro work as well as validate the key predictions of the proposed learning rule put forth in the RDE learning rule. While it is not yet clear the exact mechanisms by which ACh trains V1 to produce reward timing activity, ACh has also been shown to affect change within mouse primary auditory cortex (Letzkus et al., 2011; Guo et al., 2019) and is thought to engender such changes via disinhibition (Letzkus et al., 2015). Regardless, these results describe the powerful role that ACh has in V1 learning to produce reward timing activity.

Although the above results lay a strong theoretical and experimental foundation to understand how V1 learns to produce reward timing, it is unclear how, once learned, V1 produces reward timing activity. Specifically, how V1 circuitry affords it the ability to produce reward timing in its canonical forms (SI, SD, and P) is heretofore unknown. The previously-described computational model (as schematized in Figure 1A), produces reward timing only with the SI form of reward timing. How is it that V1 produces reward timing in the other forms? Returning to a computational approach, we ask what minimal addition/s to the RDE provide/s a means to generate the heterogeneity of response forms?
To address the ability of V1 to produce reward timing in the various forms, researchers incorporated inhibitory interneurons within this network architecture (Huertas et al., 2015). To start, the network of excitatory cells received broad and sparse inhibition (Figure 2A); specifically, excitatory cells were unlikely to receive any inhibition (“sparse”) and those with inhibition had a wide range of inhibitory strengths (“broad”). This network then underwent training with the RDE learning rule and reward timing was expressed by the network in the three canonical forms (Figure 2A) demonstrating that a simple connectivity rule plus the RDE learning rule is sufficient to express reward timing with all three canonical forms. To achieve a greater understanding of the circuit architecture, negligible synaptic weights were then pruned away from the overall structure to reveal a core network architecture (schematized in Figure 2B) This network architecture is composed of four populations of neurons: three excitatory and one inhibitory population. These four populations differ in their relative amount of recurrent excitation, non-recurrent excitation, and inhibition. When this core network architecture was trained on the RDE learning rule, it was found that reward timing activity was produced in all forms. Specifically, the three
excitatory populations produce reward timing in all forms and the single inhibitory population produced reward timing with only the sustained increase form.

In this way the core network architecture is a hypothesized network architecture that may exist within V1 to produce reward timing. Just as the original theoretical work created implications for the proposed reinforcement signal that were followed by experiments involving the basal forebrain cholinergic system, this theorized network architecture also allows for experimental testing of implications. Specifically, the model has, at least, two testable implications: (1) inhibitory interneurons represent time predominantly as the SI form and (2) neurons inhibited by interneurons represent time predominantly as the SD or PK form. In this way, the core network architecture provides a computational solution for how a network of cells produces reward timing activity with a diversity of response types. Specifically, it posits that with a simple learning rule coupled to a simple connectivity rule, a network can learn not only to associate arbitrary inputs with the rewarding outcomes but also produce activity that relates to the interval between the two events. Additionally, the inclusion of the connectivity rule affords the ability to produce timing with the three canonical reward timing forms. Furthermore, as the components of the network (e.g., GABAergic interneurons and excitatory cells) are present throughout the brain, it puts forth V1 as a possible site of the theoretical architecture allowing for biological experimentation to investigate the model’s implications. However, while this model makes the simplifying assumption that V1 interneurons are a homogenous population, it is known that there is a heterogeneity of interneurons within V1 and it is unclear how this biological diversity intersects with the model’s predictions. In future chapters, I will put forth evidence that V1 produces reward timing activity in a manner consistent with the core network architecture and that subpopulations of interneurons differentially aid in the production of reward timing.
This ability to go between computational and biological experiments within neuroscience to create and then test predictions about the capabilities of neural circuits has been an effective strategy for elucidating circuit mechanisms underlying complex response patterns. While we have used this strategy here to deepen our understanding of V1 reward timing activity, it is important to note that this strategy may become more relevant and useful as recording technology advances to aid in the recording, across days, of large and stable neuronal ensembles.

1.3: Effective Strategies to Study Circuit Mechanisms

As described above, many primary sensory areas undergo changes following associative learning that reflect the learned relevance of stimuli. While it is relatively easy to report the existence of such modulations, it is more complicated to understand the means by which these areas produce and learn to produce such responses (so-called “circuit mechanisms”). Furthermore, recent technological advances have allowed for the ability to record from hundreds of neurons within an area or across areas (Sofroniew et al., 2016; Jun et al., 2017) and allowed for direct recordings of both neurotransmitters (Marvin et al., 2018, 2019) and neuromodulatory systems (Jing et al., 2018; Patriarchi et al., 2018; Sun et al., 2018) via fluorescent reporters. With such advents, the size and parameter space of these datasets has greatly expanded to a near overwhelming scope. How can one understand circuit mechanisms in light of such advances and what might be effective strategies to gain insight into such network activity patterns? As has been demonstrated with V1 reward timing activity, an interplay between computational and experimental work is one such strategy that allows for effective understanding of neural activity.
Following its initial description, V1 reward timing activity was modeled as a recurrent network of excitatory cells with a simple learning rule (Gavornik et al., 2009). This learning rule posited, among other things, that a reinforcement signal was required to train the network to produce reward timing activity. Though, the original computational work did not address which of many possible neuromodulatory systems may provide the purported reinforcement signal, experimentalists were able to take advantage of hypothesized roles of the basal forebrain cholinergic system (Richardson and DeLong, 1988). Such hypothesized roles coupled with extensive cortical arborization (Wu et al., 2014; Zaborszky et al., 2015) made BF-ACh a prime candidate for the theorized reinforcement signal. As such, experimentalists were able to narrow their focus of all possible neuromodulatory systems to implicate and define the basal forebrain cholinergic system as a necessary and sufficient reinforcement signal for V1 reward timing activity (Chubykin et al., 2013; Liu et al., 2015).

Again, we are able to take a similar strategy to understand not just how V1 learns reward timing activity, but also how it produces reward timing activity. Recently, a network architecture has been proposed that produces reward timing activity (Huertas et al., 2015; Figure 2). In this architecture, the various forms of reward timing activity arise due to the interactions between excitatory and inhibitory neurons. The diversity of cell types within V1 has been well established. For instance, V1 interneurons fall mainly into one of three subpopulations expressing either parvalbumin (PV), somatostatin (SOM), or vasoactive intestinal polypeptide (VIP) (Xu et al., 2010; Tremblay et al., 2016). Each are unique in their connectivity patterns (Pfeffer et al., 2013) and are functionally distinct during stimulus representation in V1 (Atallah et al., 2012; Lee et al., 2012; Wilson et al., 2012). Although, the model takes the simplifying assumption that interneurons are a single population, the proposed circuit architecture allows for experimentalists to address whether its
implications are borne out *in vivo* and, if so, how the diversity of interneuron subtypes intersects with these implications. As was the case in earlier computational work, experimentalists can address how the model implications intersect with known interneuron diversity as it relates to previously described anatomical and functional differences.

Instead of relying on computational work to guide biological experiments, one can imagine recording widely from neurons across a number of brain regions without a computational scaffolding. Although such a strategy provides a wealth of data, the interpretation of such data becomes difficult. Without the context provided by computational models, it may be challenging to interpret what the salient aspects of recordings are. Additionally, computational models may also be used to guide experimentalists in understanding which recording technique is ideal for addressing a given question. For instance, if spike timing is thought to be essential based on theoretical work, experimentalists would be hard-pressed to uncover such intricacies using standard calcium-imaging protocols. Of course, such open-ended, hypothesis-free explorations of neural activity can prove useful. This is especially the case when a computational model provides predicted outcomes, but biological data reveal unpredicted response patterns or properties. In this way, circuit mechanisms are well understood when computational and experimental approaches are used in an iterative strategy. Computational work provides implications and testable hypotheses and experimental work allows for the testing of such implications while also providing more insight as to the biological instantiation of the hypothesized network and/or learning rules. As the scope and size of neuroscience datasets continues to increase, such a strategy may prove useful in defining circuit mechanisms.

In this chapter, I have described the history of V1 reward timing activity and the evidence that implicates V1 as a substrate for learning this activity. Previous work posits that V1
learns this activity in a manner similar to the proposed RDE learning rule using acetylcholine release from the basal forebrain as a reinforcement signal (Gavornik et al., 2009; Chubykin et al., 2013; Liu et al., 2015). Previous work also shows that reward timing activity comes in three main forms (as a sustained increase of activity, a sustained decrease of activity, or a peak of activity) that reflect the time to an upcoming reward (Shuler and Bear, 2006; Chubykin et al., 2013; Liu et al., 2015). How these forms come about in V1 is unknown, but a computational model posits that the heterogeneity of responses is the result of a simple learning rule combined with a simple connectivity rule (Huertas et al., 2015). I have also argued that such a strategy (iteratively using computational and biological experiments to define circuit mechanisms) is an effective strategy and should be taken into account as the breadth and magnitude of datasets continue to increase.

In subsequent chapters, I will first provide further evidence that V1 is a source of reward timing activity through a thorough characterization of reward timing activity in V1 of head-fixed mice (Chapter 2: Interval Timing in the Primary Visual Cortex of Head-Fixed Mice). In the same chapter, I will also describe our investigations of long-latency responses following alternative conditioning strategies. Additionally, through optogenetic identification of circuit components, I will show that V1 neurons express reward timing in a manner consistent with the theorized core network architecture (Chapter 3: Optogenetic Identification and Perturbation of V1 Reward Timing Activity). Finally, I will combine the data presented here to address contemporaneous issues within systems neuroscience as well as propose future experimentation that will further our understanding of V1 reward timing activity (Chapter 4: General Discussion and Potential Future Directions).
V1 reward timing activity, like many complex responses in the brain, requires the coordinated activity of many cells within a network. By furthering our understanding of how this network produces this activity pattern, we may hopefully glean general principles by which the brain is able to represent, and update representations, of the external world.
Chapter 2: Interval Timing in the Primary Visual Cortex of Head-Fixed Mice

V1 reward timing activity is a specific example of interval timing activity as this neural activity is thought to represent the time to expected reward between a visual stimulus and an upcoming water reward. As described in the previous chapter, reward timing activity in V1 comes in three forms and depends on acetylcholine (ACh) released locally within V1 from basal forebrain (BF) innervation. This reward timing activity has been previously described in freely-moving rats (Shuler and Bear, 2006; Chubykin et al., 2013); additionally, although behaviorally-conditioned reward timing activity in mouse V1 has been reported (Liu et al., 2015), it has not been fully characterized (as that previous work focused on whether conditioning with ACh-fiber activation was sufficient to give rise to cued-interval timing within V1). Specifically, as it relates to mouse V1 reward timing, it is not known whether reward timing in mouse V1 expresses this activity in the three canonical forms or whether this activity can reflect various conditioned intervals, among other open questions. Furthermore, as it relates to V1 reward timing, generally, much work has been done to understand how V1 as a network learns to produce reward timing activity in the observed forms, but little is known how, once learned, V1 produces this reward timing activity in the three canonical forms. In order to gain a better understanding of the ability of mouse V1 to produce reward timing activity, the overall parameter space of V1 reward timing activity, and how V1 produces reward timing activity in the three forms, a thorough characterization of reward timing activity in mouse V1 is required. Efforts relating to such a characterization are described in Section 2.2: Reward Timing in Mouse V1.

V1 reward timing activity is a specific example of interval timing activity which, itself, is a specific version of what can be referred to as persistent activity or long-latency responses.
The main difference between instances of interval timing and persistent activity is that even though in persistent activity, one may be able to define a time course of the neural activity and create some distribution of a timed value, the timing itself is not reflective of the interval to an external event; whereas in interval timing, there is a relationship between the time course of the neural activity and the animal’s environment. To differentiate between these two phenomena, it is necessary to determine whether the prolonged activity corresponds with the delay of interest (as is the case in interval timing activity). Additionally, it is necessary to test for this relationship across at least two conditioned intervals and determine if a change in the temporal association results in a concomitant change in the neural activity (as is required of an interval timing signal). For instance, in previous reports of V1 interval timing activity, the persistent activity exists as a representation of the time between a visual stimulus and a reward or between a visual stimulus and optogenetic activation of a reinforcement signal (Shuler and Bear, 2006; Chubykin et al., 2013; Liu et al., 2015). Furthermore, this activity has been shown to update when the conditioned interval is changed (Shuler and Bear, 2006; Chubykin et al., 2013; Liu et al., 2015). However, it is unclear whether there is persistent activity in other conditioning strategies and whether this persistent activity may be classified as examples of interval timing. In Section 2.3: V1 Long-Latency Responses in Other Behavioral Tasks, I will detail the existence of persistent activity and determine whether these results accord with other results of interval timing activity.

Specifically, little is known about potential “after-effects” of V1 neurons after a transient visual stimulus is delivered. Cue-evoked persistent activity in the primary visual cortex prior to associative learning has been observed as sustained membrane depolarization in mouse V1 (Funayama et al., 2015, 2016), emitted spikes in mouse V1 (Funayama et al., 2015), and complex V1 LFP responses in the mouse (Funayama et al., 2016), rat (Kimura,
1962; Zold and Hussain Shuler, 2015) and rabbit (Bishop and O’Leary, 1936). Specifically, when rats are trained on a reward timing task, LFP theta oscillations persist beyond the envelope of visual stimulation early in training in a manner that accords with the strength of the visual stimulus (Zold and Hussain Shuler, 2015), only to subsequently converge toward the cue-reward interval after animals associate the cue to the delayed reward. Additionally, whole-cell patch clamp recordings of L2/3 neurons within V1 show evidence of prolonged membrane depolarization which can result in delayed spiking activity up to hundreds of milliseconds following a single visual flash (Funayama et al., 2015, 2016; Minamisawa et al., 2017). It is not clear if these prolonged responses (a) exist within single neurons in deeper layers of V1 or (b) what form(s) these responses take, if they do exist.

In Section 2.3.1: Responses to Visual Stimuli in the Absence of an Unconditioned Stimulus, I describe the distribution of long-latency responses observed in V1 when monocular visual stimuli are repeatedly presented in the absence of a US.

Little is known about long-latency activity outside of reward conditioning. This includes responses to unpaired visual stimuli prior to any conditioning as described above, but it also includes possible long-latency responses following other conditioning strategies. Specifically, we sought to determine if any outcome may engender interval timing activity within V1. As described in the previous chapter, a neuron need only access to the conditioned stimulus and a reinforcement signal to produce interval timing activity. The purported reinforcement signal is carried by cholinergic neurons in the basal forebrain which are known to respond strongly to both positive and negative reinforcers (Hangya et al., 2015; Guo et al., 2019) and relatively weakly to conditioned stimuli (Guo et al., 2019). Further, direct activation of cholinergic fibers within V1 results in the production of long-latency responses timed to the delayed interval (Liu et al., 2015). As such, we tested the hypothesis that aversive conditioning, but not neutral conditioning, would engender timing
activity within V1. Surprisingly, we find that though neurons recorded during these conditioning strategies can express long-latency responses, the responses are unable to track the conditioned interval as had been seen during reward conditioning. These experiments are described within Sections 2.3.2: Neutral Conditioning Does Not Engender Interval Timing Activity and 2.3.3: Aversive Conditioning Does Not Engender Timing Activity.

Finally, this cued-interval timing activity has been shown to be behaviorally relevant in the rat (Namboodiri et al., 2015), but it is unclear to what extent these responses track animal behavior on a trial-by-trial basis within the head-fixed mouse. To this end, we have developed a novel action timing task for the head-fixed mouse and have seen that a portion of responses do, in fact, track the animal’s time to first lick on a trial-by-trial basis. These results are described in Section 2.3.5: Action Timing for the Head-Fixed Mouse.

Together, the results presented in this chapter provide greater detail into the production of V1 interval timing activity (specifically, reward timing activity). Such detail is useful when investigating the circuit mechanisms underlying this activity as will be described in subsequent chapters.

2.1: Materials and Methods

All procedures performed on animals were in accordance with the US NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the Johns Hopkins School of Medicine.
2.1.1: Animal Information and Surgical Procedures

Male mice (N = 31, between 2 and 6 months old) were used for the experiments described in this chapter. The number and genotype of animals used varied based on the conditioning strategy and, for ease, they will be described separately for each behavioral task in the respective Behavioral Task Design section. Surgical procedures, however, were largely consistent across all conditioning strategies (described below).

Surgical procedures were performed under aseptic conditions and were in accordance with the Animal Care and Use Committee at the Johns Hopkins School of Medicine. Animals underwent two surgeries spaced at least two weeks apart from one another. Prior to either surgery, mice were anesthetized using a cocktail of ketamine (Ketaset, 80mg/kg) and xylazine (Anased, 10 mg/kg) and eyes were covered with ophthalmic ointment (Puralube). The first surgery was performed to affix a head-restraint bar to the animal’s skull for training purposes and to mark sites for future craniotomies. In the first surgery, the hair covering the skull was removed (Nair), the skin cleaned with alternating 70% ethanol and iodine, then the skin was cut away. Following this, the periosteum was removed and the skull cleaned with alternating 70% ethanol and hydrogen peroxide, then the skull was dried with canned air. A total of four sites were marked for future craniotomies: two for ground screws (arbitrarily marked over the anterior parietal bone) and two for primary visual cortex (measured as 3mm lateral to lambda, bilaterally). Sites for future craniotomies were covered in a silicone elastomer (Smooth-On Body Double) and a head-post was affixed to the anterior portion of the mouse’s skull with super glue (Loctite 454). The remaining bone was covered in super glue. A second surgery was performed to implant recording electrodes. Briefly, small craniotomies were made using a dental drill for ground screws and screws were implanted into sites. Next, craniotomies were created over V1, the dura was cleaned with sterile paper points, and electrodes were
brought to the surface of the brain, then implanted 500μm below the cortical surface in accordance with stereotaxic measurements of V1 (Franklin and Paxinos, 2008). Wires were covered in sterile ophthalmic ointment (Puralube) and encased in dental cement (Orthojet). Ground screws and ground wires were connected and a headcap was built of dental cement.

2.1.2: Behavioral Task Design – Reward Timing Tasks

For reward timing experiments described in 2.2: Reward Timing in Mouse V1, we used a total of 18 mice from four genetic backgrounds which differ in their expression of the light-activated cation channel, channelrhodopsin-2 (ChR2). One cohort expressed no ChR2 (wildtype or WT, n = 7 animals), one cohort expressed ChR2 in parvalbumin-positive interneurons (PV-ChR2, n = 4 animals), one cohort expressed ChR2 in somatostatin-positive interneurons (SOM-ChR2, n = 4 animals), and one cohort expressed ChR2 in vasoactive-intestinal-polypeptide-positive interneurons (VIP-ChR2, n = 3 animals). The WT cohort was composed of C57BL/6 mice (Strain Code: 027, Charles River Laboratories). ChR2-expressing mice were derived by selectively breeding the following genetic lines: a parvalbumin-Cre recombinase line (PV-Cre; 008069, Jackson Laboratory, (Hippenmeyer et al., 2005)), a somatostatin-Cre recombinase line (SOM-Cre; 013044, Jackson Laboratory, (Taniguchi et al., 2011)), a vasoactive-intestinal-polypeptide-Cre recombinase line (VIP-Cre; 010908, Jackson Laboratory, (Taniguchi et al., 2011)), and a loxP-STOP-loxP-channelrhodopsin-2-eYFP Cre-dependent line (ChR2-eYFP, Ai32; 012569, Jackson Laboratory, (Madisen et al., 2010)). The mice of these crosses were on mixed backgrounds composed primarily of C57BL/6 and CD-1. All mice from all cohorts underwent identical training and training occurred in the light cycle during a 12h light/dark schedule (lights provided between 0700 and 1900).
Prior to electrode implantation (between the first and second surgeries), animals were habituated to head-fixation over the course of 2-3 days, and then were trained that a visual stimulus predicted a water reward at a fixed delay for 2-3 weeks. Visual stimuli were full-field retinal flashes delivered monocularly to the left (Cue 1) or right eye (Cue 2) via head-mounted goggles. These goggles are custom made and consist of a miniature LED glued to the back of a translucent, plastic hemidome. Licks were recorded on a lickometer via an infrared beam break (IslandMotion); experiments were controlled through an Arduino Mega microcontroller board (Arduino) and events were recorded with Neuralynx. In every session, trials were separated by an inter-trial interval (ITI, between 3 and 8 seconds, uniformly distributed). In order to initiate the next trial (and exit the ITI), animals had to cease licking for a random interval during the later portion of the ITI (deemed a “lick lockout”). This lick lockout period was the same across conditioned delays and was used to discourage non-stimulus-evoked licking, as licks within this period caused the timer to restart and, thus, a longer ITI. Upon trial initiation, a monocular visual stimulus was either delivered (CS trials) or withheld (Sham trials), followed by a delay window. CS’s were visual stimuli which lasted 100ms and were delivered, with equal probability, to the left or right eye. The delay to reward was the same for both CS’s within a session and was held constant for several consecutive sessions as either the short (1s) or the long (1.5s) delay. Sessions conditioned with the short delay constitute the “Short Delay Sessions”; those with the long delay, the “Long Delay Sessions”. CS trials were further divided into “paired” and “catch” trials; paired trials being trials in which a small water reward (~2μL) became available following the delay period, provided that the animal made at least one lick on the lick port within the delay. Catch trials, however, were trials in which the reward was withheld regardless of behavior. Licks were never rewarded during Sham trials. At the conclusion of the delay window on both CS and Sham trials, the animal re-entered the ITI. Trials in which the animal licked during the delay window are defined as “Hit” trials and
trials in which the animal did not lick during the delay window are defined as “Miss” trials. Unless otherwise noted, data presented here are from Catch+Hit trials (i.e., trials in which the animal received a visual stimulus, licked during the delay window, and did not receive a water reward at the end of that delay). The relative proportion of paired/catch trials and sham trials was systematically varied across behavioral shaping as well as the requirement to lick within the delay window. In the final form of the task (and in all sessions reported here), 80% of trials were CS trials (with equal probability of being paired or catch), with the remaining trials being Sham trials. A cartoon of appetitive conditioning is shown in Figure 3.

A subset of WT animals (n = 4) underwent Extra Short conditioning sessions which was identical in nature to the task described above except that the conditioned interval was 500ms following visual stimulus offset.

2.1.3: Behavioral Task Design – Unpaired Visual Stimulus Presentations

10 animals experienced unpaired visual stimuli while neural activity was recorded from V1. These animals were all WT animals as described above (2.1.2: Behavioral Task Design – Reward Timing Tasks). Following electrode implantation, animals recovered from surgery for 5 – 7 days after which they were habituated to head fixation over the course of 2 days before experiencing unpaired visual stimuli. This “pseudo-conditioning” is similar to reward conditioning in most manners with the exception that at the end of a “delay window” there is no reward delivered. No outcome was delivered following monocular visual stimulation and no licks were required nor recorded. Animals underwent 3 – 5 days of pseudo-conditioning prior to moving on to either Neutral or Aversive conditioning (described below).
2.1.4: Behavioral Task Design – Neutral Conditioning

7 animals underwent Neutral Conditioning (4 of which underwent neutral conditioning following several days of pseudo-conditioning described above). This task is similar to reward timing task with the exception that instead of a water reward at the end of a delay, the animal received a binocular visual stimulus via the head-mounted goggles. No licks were required to receive the binocular visual stimulus nor were licks recorded. Upon neutral conditioning initiation, animals were shaped in neutral conditioning in a manner similar to shaping for the reward timing task. Specifically, over the course of several days, the delay was initially short and was prolonged to 1 and then 1.5 seconds and the relative proportion of paired/catch trials was changed until task parameters matched those of the reward timing task (described above).

2.1.5: Behavioral Task Design – Aversive Conditioning

6 animals underwent aversive conditioning following several days of pseudo-conditioning. Following pseudo-conditioning sessions, animals were taken to a separate recording room and were habituated to wearing tail cuffs that would provide the aversive tail-shock (screw terminals connected to a stimulus isolator). After habituation, responses to unpaired shocks were recorded to parameterize an appropriate magnitude for the tail shock (Figure 13). After parameterization, animals were taken back to the original recording room and underwent shaping for aversive conditioning. Shaping for aversive conditioning matched shaping for neutral conditioning as described above. A flow chart describing the animals used for pseudo-conditioning, neutral conditioning, and aversive conditioning is shown in Figure 4.
Figure 3: Reward timing task cartoon. Shown is a schematic of trial types within the task. After an animal exits the ITI it either enters a CS (top) or Sham trial (bottom). In CS trials, a CS is delivered monocularly to the left eye. Delivery of the CS starts a delay window of 1 (short) or 1.5 (long) seconds. If an animal licks during the delay (CS+Hit Trials), it will receive a reward on Paired trials. However, if the animal does not lick during the delay window (CS+Miss Trials), the animal will not receive any reward. Alternatively, once an animal exits the ITI, a sham trial may initiate. In this trial type, no CS is delivered but the delay window is again started after trial initiation. Regardless of licking behavior, the animal will not receive a reward in Sham trials. For consistency, sham trials in which the animal licks within the delay are referred to as Sham+Hit trials and sham trials in which the animal does not lick are referred to as Sham+Miss trials.
2.1.6: Behavioral Task Design – Action Timing Task

Two PV-ChR2 animals underwent action timing training. As was the case in animals that experienced the reward timing task, these animals were trained prior to electrode implantation. In the final form of the task, the animals received a binocular visual stimulus and were required to wait to make a lick on a lick port. The longer the animal waited, the larger the reward it received, up until 1s (after which there was a steady decline of reward volume that became unavailable following 3s). If animals licked prior to 500ms post-visual stimulus onset, no reward was delivered. A task schematic is shown in Figure 16. Animals were shaped on this task by systematically shifting of the minimum wait time (500ms in final version), the relationship between waiting and volume received, and the maximum reward time (1s in final version).

2.1.7: Behavioral Measurements

For animals that experienced either the reward timing task or the action timing task, the timing of individual licks was recorded using a lickometer (IslandMotion) and were
recorded simultaneously with neural data. To quantify licking behavior in the reward timing task we took advantage of the fact that animals tended to make one lick bout following delivery of the CS. As such, the timing of this bout is quantified as the time of the first lick within the bout, the time of the last lick within the bout, and the mean time between these two licks (“Bout Midpoint”). For the action timing task, we used the time to the animal’s first lick as the behavioral readout.

2.1.8: Electrophysiology

For all animals across all conditioning strategies, neural activity was recorded bilaterally from primary visual cortex using custom-built recording electrodes. Per recording electrode, 16 channels of neural data were recorded at a sampling rate of 32,556 Hz through commercial hardware (Neuralynx). Neurons were offline identified through manual 3D cluster-cutting methods through commercial software (Offline Sorter, Plexon). Electrodes composed of a connector with 16 recording channels and two ground wires (Omnetics). Bundles were cut at a ~45° bias to allow for sampling across a depth of approximately 250 microns. Additionally, for animals which experienced the reward timing task or the action timing task, an optic fiber was affixed next to the bundle of wires; details of this fiber and optogenetic stimulation are described in the following chapter (Chapter 3: Optogenetic Identification and Perturbation of V1 Reward Timing Activity).

2.1.9: Neural Data Analysis – Interval Timing Classification

The form with which a neuron expressed long-latency responses was determined using manual classification in a blinded fashion. Specifically, a neuron was randomly selected from a random session. Then, a peri-stimulus time histogram (PSTH) calculated from trials that were either Cue 1 Catch+Hit trials, Cue 2 Catch+Hit trials, or Sham+Hit trials was
randomly presented. This PSTH was then classified as “Not Classified” (NC), “Sustained Increase” (SI), “Sustained Decrease” (SD), or “Peak” (SD). The remaining PSTH’s were presented, followed by the remaining neurons. These classifications were performed without knowledge of animal identity, recording session, delay time, or conditioning strategy.

2.1.10: Neural Data Analysis – k-Nearest Neighbors Classification

Within responses recorded during the reward timing task, we sought to cross-validate the human classification of reward timing neurons. To do so, we implemented the supervised learning algorithm k-Nearest Neighbors (kNN). Briefly, kNN takes classified data as a “training example” to then determine the identity of unclassified “query points” based on the proximity of query points to classified training example points. Identity of the query point is defined as a plurality vote of its k nearest neighbors in the training example. In our case, we first split data from reward timing neurons into two halves: neural activity from even trials and neural activity from odd trials. Then, we normalized neural activity using the area under the ROC curve (AUC, see below) and used principal components analysis (PCA) for dimensionality reduction. Specifically, we reduced the normalized firing activity from even trials to the first eight principal components which explained >85% of the variance within the neural activity; the projection in eight dimensions and human-classified identity of the responses recorded in even trials served as the training example for the kNN classifier. Then, data from the odd trials were projected into the 8-dimension subspace (acting as the query points) and were classified across a range of k. Specifically, we varied the number of neighbors between 1 and 65; to avoid ties, we only used an odd number of neighbors in our classification.
2.1.11: Neural Data Analysis – Neural Report of Time Calculation

To attribute a time to the long-latency responses recorded from neurons in the various conditioning tasks, we calculated the Neural Report of Time (NRT). The NRT is the moment taken as the time which neurons return to a baseline level of activity, for SI and SD response forms, or the time of maximum firing rate from baseline (after the visual-evoked response), for the PK response form.

To calculate such a time, neural activity was normalized to the baseline firing rate by calculating the area under the ROC curve (AUC) using a sliding 100ms window (Cohen et al., 2015; Sadacca et al., 2018). An AUC value of 0.5 means that the ideal observer would be at chance level to tell apart two distributions and values above or below 0.5 reflect greater dissimilarity among two distributions. For our purposes, we found the AUC value between the distribution of spike counts from a 100ms window of baseline pre-stimulus activity, and a given 100ms of spiking activity across all trials of the same type (e.g., Paired, Cue 1 trials; Catch, Cue 1 trials; etc.). In this way, we do not rely on the averaging of spike counts in the same way that a PSTH does and thus the resultant value is more robust against a small subset of trials with many spikes or other forms of inter-trial spiking variability. Furthermore, this method normalizes the firing rate to a value bounded by 0 and 1 for every set of trials. As the AUC-normalized firing rate is the magnitude of difference and not the sign of the difference between an AUC value and 0.5 (which determines how dissimilar two distributions are), we found the absolute value of the difference between the AUC vector and a value of 0.5. In doing so, neurons with sustained activation or suppression (SI or SD neurons, respectively) could be treated with the same algorithm to calculate an NRT. We operationally defined a difference threshold of 0.15 (true AUC value of 0.35 or 0.65), and, using this threshold, we then defined the NRT as the first moment in time when the AUC difference vector fell below the threshold for at
least 100ms. For classified PK neurons, the NRT was defined as the time of the maximum of this AUC difference vector. To avoid conflating timing responses with general visual responses, we set a minimum value for valid NRTs as 0.5s after stimulus offset.

2.1.12: Neural Data Analysis – Calculation of ΔSpikes

This value is used to determine the average change in spike rate based on an animal’s first lick within Appetitive Conditioning. It is defined as follows:

$$\Delta \text{Spikes} = \frac{\text{Spikes}_{\text{pre}} - \text{Spikes}_{\text{post}}}{N}$$

Where $\text{Spikes}_{\text{pre}}$ is the number of spikes in the 100ms preceding the first lick within a Sham+Hit trial, $\text{Spikes}_{\text{post}}$ is the number of spikes in the 100ms following the first lick within a Sham+Hit trial, and $N$ is the number of trials of Sham+Hit trials within the session.

2.1.13: Neural Data Analysis – Calculation of J3 Statistic

This statistic was developed to determine whether neurons are the same from one recording session to the next (Moran and Katz, 2014). First the waveforms of all spikes recorded from two recordings are projected onto reduced dimensions using PCA. Then, values are calculated as follows:

$$J_1 = \sum_k \sum_{k_i} \|s_{k_i} - m_k\|^2$$

$$J_2 = \sum_k N_k \|m_k - m\|^2$$

$$J_3 = J_2 / J_1$$

Where $s_{k_i}$ is the projection in two dimensions of spike $i$ in session $k$, $m_k$ is the mean vector of all spikes ($N_k$) from the $k^{th}$ session, $m$ is the overall point mean of the projection, and $\|\cdot\|$ represents the Euclidean Distance. In essence, the J3 value is a ratio between the
Euclidean distance between each spike’s waveform and the center of the cluster of all other spikes’ waveforms from that neuron to the distance between the two clusters (i.e., a ratio of the inter- and intra-cluster distance). J3 is maximal when two recordings are tightly packed and far away from one another in PC space; this reflects that two recordings are unique from one another. However, we wished to utilize this statistic to determine whether a neuron recorded on one day was the same as a recording made on the same channel the subsequent day. To do so, we defined a J3 threshold by finding all “within” J3 values (that is, the J3 value between the first third of the recording’s spikes and the last third of the recording’s spikes). The threshold was defined as the 95th percentile of this distribution. That is to say, any neurons which were recorded from the same animal and on the same channel which had a J3 value that was less than this threshold was deemed the same.

2.1.14: Neural Data Analysis – Similarity Measurements of Reward Timing Responses

Within the animals undergoing reward timing training, we wished to assess the similarity of reward timing responses of a given neuron across the two CS’s to assess the consistency of reward timing responses when different cues predicted the same reward occurring at the same delay. Furthermore, where possible, we wished to assess the stability of a neuron’s reward timing response to the same stimulus across sessions. Reward timing responses of a neuron could differ (or persist) between cues or across sessions in their presence, form, timing, and shape. For instance, within a session, reward timing responses may be present within a given neuron to both cues, exhibit the same response form (e.g., SI) with an overall similar response shape, and report nominally similar NRT’s. Additionally, neurons can express similar responses to the same stimulus across days. To determine how similar these responses are, we first calculated the
concordance of reward timing forms (for example, how often a SI cell expresses reward timing as SI for the opposite CS or on a following day). Among the responses which are concordant, we then determined the similarity in the neuron’s report of time by calculating the absolute difference in NRT’s. Finally, within these responses, we quantified the similarity in shape by calculating the Euclidean distance between the evoked responses. These values were compared with a shuffled control distribution. Shuffling distributions were calculated by shuffling across neurons that expressed reward timing in the same form for the same conditioned interval.

2.1.15: Histology
Animals were deeply anesthetized using sodium pentobarbital (200mg/kg, Vedco). After which, animals were transcardially perfused with ice cold phosphate-buffered saline (PBS) followed by ice cold 4% paraformaldehyde (PFA). Brains were immersion fixed overnight in 4% PFA and were transferred to 30% sucrose until sectioning. Brains were sectioned on a cryostat into 60μm slices. Electrode location was verified using Nissl staining, as follows. Sections containing V1 were selected and mounted on gelatin-subbed slides and air dried. These slides were then immersed in a solution containing 0.1% Cresyl violet and 1% glacial acetic acid dissolved in water for 5 minutes, followed by a 2-minute wash in distilled water, then by 2 minutes in 50% ethanol, then 2 minutes in 70% ethanol. Stained and washed sections were air dried, immersed in xylenes then coverslipped with Permount Mounting Medium (Electron Microscopy Sciences).

2.2: Reward Timing in Mouse V1
The ability for mouse V1 to produce behaviorally-conditioned reward timing activity has been previously reported (Liu et al., 2015); however, an in-depth characterization of this
neural activity is lacking. Such a characterization would provide insight as to how V1, as a whole, produces reward timing activity in the various forms observed previously (Shuler and Bear, 2006; Chubykin et al., 2013). Here, I describe such an in-depth characterization of behaviorally conditioned reward timing activity within V1 of the head-fixed mouse.

2.2.1: Head-Fixed Mice Learn Reward Timing Task

Mice were trained to associate a water reward with visual stimuli (see 2.1.2: ). Briefly, head-restrained mice received a 100ms visual stimulus delivered to the left or right eye with equal probability (Cue 1 and Cue 2, respectively) via head-mounted goggles and received water from a lick port placed within reach of the tongue. Trials were initiated after a lapse of time comprising a randomly selected interval and a second random interval less than the ITI during which the animal must not lick (a “lick lockout” interval). If an animal licked during this lick lockout, the lockout timer would restart. Such an ITI encourages mice to cease licking and to wait for the onset of the next trial. Upon the initiation of a trial, animals received a monocular visual stimulus delivered to the left or right eye with equal probability, after which the animal was required to make at least one lick within the subsequent delay period so that reward could be delivered at the end of the delay. On half of these trials, if the animals met this behavioral requirement, they received a small water reward (~2µL) at the end of the conditioned interval (so-called “paired” trials). On the other half of these trials, regardless of lick behavior, reward was withheld (“catch” trials). On 20% of trials, neither a visual stimulus nor a reward were delivered although the intertrial interval and lick lockout periods expired successfully; these trials are referred to as “sham” trials and are used to verify that animals are using visual stimuli to guide licking behavior (as opposed to timing lick bouts from events other than a visual cue). Regardless of trial type, trials in which the animal made a lick during the delay window are defined as Hit
trials and trials in which the animal did not lick were referred to as Miss trials. All data presented here, unless otherwise noted, are from Catch+Hit trials (i.e., trials in which the animal received a visual stimulus, licked during the delay, and did not receive a reward). A cartoon of possible trial trajectories is shown in Figure 3.

The delay time used was the same for both visual stimuli within a recording session and varied across days, as follows. On Short Delay Sessions the delay time was 1 second following visual stimulus offset, and on Long Delay Sessions the delay time was 1.5 seconds following visual stimulus offset. A task schematic is shown in Figure 5A and behavior from an example session is shown in Figure 5B.

As expected, animals showed a high probability of licking in the delay period on trials where a light stimulus was delivered (“CS trials”) and a low probability of licking during the sham trials (70.67% and 14.04%, respectively, Figure 5B and Figure 5C). There is a significant effect of trial type (i.e., CS trial vs sham trial, $\chi^2(1, 286) = 464.11, p = 7.83 \times 10^{-62}$, Kruskal-Wallis test) on the probability that an animal licks while there is neither a significant effect of session number nor a significant interaction (Session Number: $\chi^2(8, 286) = 0.51, p = 0.85$; Interaction: $\chi^2 (8, 286) = 1.26, p = 0.26$, Kruskal-Wallis test). These results demonstrate that animals lick in response to reward-predicting visual stimulation and that their behavior had reached asymptotic performance at the time of recording.
Figure 5: Head-fixed mice learn reward timing task. (A) Animals were trained that a monocular visual stimulus (the conditioned stimulus, CS) delivered to the left or right eye predicted a water reward (the unconditioned stimulus, US) at a fixed delay. Upon receipt of the CS, animals were required to lick during the delay period so that reward could be delivered at the end of the short or long delay. Trials in which the reward is delivered are Paired trials and trials in which the reward is withheld are Catch trials. Trials in which no CS nor US were delivered are Sham trials and are used to determine the ability of the animal to use the visual stimulus to guide licking behavior. (B) Example licking behavior for a single animal during a single session of the task for all trial types with respect to the onset of the CS (time 0, green line). Blue lines represent time of reward delivery (solid line representing the receipt of reward on paired trials and dashed line representing expected time of reward on catch trials). Top: raster plot of licking behavior where black dots are licks recorded during “Hit” trials (i.e., trials in which the animal licked during the delay window) and red dots are licks recorded during “Miss” trials (i.e., trials in which the animal did not lick in the delay window). Bottom: PSTH’s calculated from licking activity, color scheme as noted above. (C) Scatter plot of session hit rates (probability of licking on a given trial) for all trials in which the CS is delivered (CS Hit Rate) and for all trials in which the CS was withheld (Sham Hit Rate) for short delay sessions (light blue) and long delay sessions (dark blue). Histograms of hit rates for each trial category shown in margins and unity line is the black, dashed line. (D) Behavioral measurements (labelled, black dashed lines) for example licking behavior during Catch+Hit trials from a short delay session (left, light blue raster) and a long delay session (right, dark blue raster). (E) Cumulative distribution plots for the three behavioral measurements measured during Catch+Hit trials during short delay sessions (light blue) and long delay sessions (dark blue). Vertical lines represent reward time and horizontal line indicates the median values. *** - p < 0.001, Wilcoxon rank-sum test.
We next addressed whether the animals are timing their behavioral response. To quantify the timing of the licking behavior, we made three measurements: the time of the first lick in a bout, the time of the last lick in a bout, and the mean time between the first and last lick in a bout (Bout Midpoint). This last measurement (the Bout Midpoint) is derived from the initiation and cessation of licking, and so is not an independent measure. Rather, its inclusion is simply to determine whether the centering of lick bouts is in good accordance with the expected time of reward. These values were recorded across trials and an average of these values were calculated for a given trial type on a given day (Figure 5D for example sessions). When we compare these values across recording days, we find that the lick initiation and cessation times (and consequently, the Bout Midpoint) are significantly smaller for short delay sessions compared to long delay sessions (Figure 5E, Mean First Licks: $Z = -6.09, p = 1.11 \times 10^{-9}$; Mean Bout Midpoints: $Z = -6.71, p = 2.01 \times 10^{-11}$; Mean Last Licks: $Z = -5.73, p = 9.89 \times 10^{-9}$, Wilcoxon rank-sum test) indicating that animals adapt their licking behavior based on the expected time to reward.

2.2.2: Neurons in Primary Visual Cortex of the Head-Fixed Mouse Express Reward Timing Activity

These behavioral data indicate that animals express an internal sense of the time interval between the visual stimulus and the water reward. To determine what, if any, neural representation of time was present in V1, we recorded single unit activity bilaterally during behavioral sessions. Previous work has shown that, in similar tasks, neurons in V1 of freely-moving rats and mice represent the time interval to an expected reward in one of three forms: a sustained increase (SI) or sustained decrease (SD) of activity until the time of reward, or as a peak (PK) of activity around the time of reward (Shuler and Bear, 2006; Chubykin et al., 2013; Liu et al., 2015). These response forms were also observed here in
the head-fixed mouse (Figure 6A). Using these response forms, we manually classified in a blinded fashion the peristimulus time histograms (PSTHs) of neurons for both Cue 1 and Cue 2 Catch+Hit trials (i.e., trials in which the animal received a visual stimulus, licked during the delay window, and did not receive a water reward at the end of that delay; see Methods). PSTHs created from Sham+Hit trials (that is, trials in which neither CS nor US was delivered, but had licks within the delay window) were also blindly classified as a control. Neurons could be classified as responsive during any of these trial types; as such, we began our analyses by quantifying “neural records” (i.e., a given pattern of activity a neuron produced during a trial type).

We recorded from 996 neurons in the primary visual cortex which yielded 1,992 neural records from Catch+Hit trials (each neuron produced two neural records: one in response to Cue 1 and one in response to Cue 2). Of these 1,992 neural records from Catch+Hit trials, 410 (20.58%) were classified as expressing reward timing (i.e., were classified as SI, SD, or PK). These 410 records were expressed by 253 neurons (25.40% of the total recorded population). Among the 410 records: 243 (59.27%) were classified as SI (47 neurons classified for one CS, 98 neurons classified for both CS’s), 105 (25.61%) were classified as SD (33 neurons classified for one CS, 36 neurons classified for both CS’s), and 62 (15.12%) were classified as PK (30 neurons classified for one CS, 16 neurons classified for both CS’s). Only 11 of 996 (1.10%) of the neural records from Sham+Hit trials were classified as one of the forms described above. Figure 6B shows the proportions of neural records classified. We also performed a cross-validation of these blindly classified responses by using a k-Nearest Neighbors (kNN) classifier (see 2.1.10: Neural Data Analysis – k-Nearest Neighbors Classification). We used neural activity from even trials as the training example and used it to classify data from odd trials. This classifier correctly identified these novel data across a range of parameters (1 ≤ k ≤ 65,
Figure 6C). By using this supervised learning algorithm, the neural activity sorted by blinded classification is cross-validated as falling into distinct classes of reward timing activity, as previously reported (Shuler and Bear, 2006; Chubykin et al., 2013; Liu et al., 2015). Throughout the text, neurons with reward timing expression to both cues will be called “binocular reward timing neurons” as opposed to “monocular reward timing neurons”; note, however, that the cues used are monocular visual stimuli and these names reflect the presence of reward timing to one or to both cues.

Figure 6 Neurons in primary visual cortex express reward timing activity in three forms. (A) Six example neurons representing the three forms of reward timing: Sustained Increase (left), Sustained Decrease (middle), and Peak (right) recorded from short delay sessions (light blue, top row) or long delay sessions (dark blue, bottom row). Activity is recorded from Catch+Hit Trials, normalized by AUC, and is plotted with respect to CS onset (green, vertical line). Dashed, vertical lines represent the time of expected reward (i.e., the conditioned interval for the session). Calculated neural report of times (NRT) for these example neurons are shown as yellow stars on x-axis. (B) Pie charts showing proportion of responses which express reward timing (left) and, of those classified responses, the proportion of the three forms of reward timing (right). (C) Performance of kNN classifier across a range of k nearest neighbors. Thick black line represents average performance of classifier, shaded region represents the mean ± standard deviation. Dashed horizontal line represents chance performance. (D) Cumulative distribution plots of the calculated neural reports of time calculated from Catch+Hit trials during short delay sessions (light blue) and long delay sessions (dark blue). Vertical lines show time of reward and horizontal line shows median values for distributions. *** - p < 0.001, Wilcoxon rank-sum test.
Previous reward timing studies have shown that V1 reward timing corresponds to the delay to reward (Shuler and Bear, 2006; Chubykin et al., 2013). By ascribing to each neuron’s classified response a “neural report of time” (NRT, the moment of time the neuron reports as the delay to expected reward, see Methods), we addressed whether timing activity to a given reward delay similarly emerges in the head-fixed mouse preparation. Should reward timing responses emerge to visual cues predicting a given delay, the central tendency of those cues’ NRT distributions should correspond to that delay. Indeed, we find that the central tendencies for the NRT distributions accord well with the conditioned intervals and are significantly different for short and long delay sessions ($Z = -4.95$, $p = 7.49 \times 10^{-7}$, Wilcoxon rank-sum test Figure 6D). Furthermore, the NRTs calculated from the cross-validated responses described above also show similar significant changes in distributions (i.e., shorter for the short delay) across the range of values for $k$ (all $Z$'s $<-3.43$, all $p$'s $<5.97 \times 10^{-4}$, Wilcoxon rank-sum test).

2.2.3: Reward Timing Activity is Not Explained by Licking Behavior

These data demonstrate that the reward timing activity we see in mouse V1 shifts in relation to the conditioned interval. But, we have also shown that animals express licking behavior based on the conditioned interval, in that they initiate and terminate licking at later times for Long Delay Sessions than they do for Short Delay Sessions (Figure 5D-E). Therefore, we sought to address what, if any, influence licking behavior has on the neural report of time. The simplest scenario is that the initiation of licking alone is sufficient to engender modulation in neural activity. However, as mentioned above, neural data recorded from Sham+Hit trials (trials in which the animal expressed non-stimulus-evoked licking after exiting the ITI) were exceedingly unlikely to be classified as expressing reward timing (1.10%, Figure 7A). This demonstrates that lick initiation, alone, is not sufficient to
engender such timed activity. Nonetheless, we sought to determine what, if any, effect lick initiation had on spiking activity in these trials. To do so, we defined “ΔSpikes” as the average change in spikes after the first lick of a Sham + Hit trial. With this variable, we find that lick initiation has no significant effect on ongoing neural activity as the distribution of ΔSpikes values did not differ from zero either across the entire population of recorded neurons (-0.004 ± 0.592 ΔSpikes, p = 0.198 Wilcoxon signed-rank test) or in neurons which express reward timing (-0.019 ± 0.814 ΔSpikes, p = 0.745 Wilcoxon signed-rank test, Figure 7B). While lick initiation is dissociable from spike modulation, might the prolonged neural activity we see be due to an interaction of cue-evoked responses and sustained licking activity? To address this, we analyzed data from two different trial types than the data described above: (1) Paired+Hit Trials and (2) CS+Miss Trials.

In Paired+Hit trials, animals received a visual stimulus, licked during the delay, and received a water reward. In such trials, the licking activity extended beyond the licking activity in Catch+Hit trials (Z = -13.34, p = 1.30 x 10^-40, Wilcoxon signed-rank test, Figure 7C), reflecting the consumption of the water reward. In contrast, CS+Miss trials are trials in which animals received a visual stimulus but did not lick during the delay window. If ongoing licking activity influences the timing of neurons with reward timing, then we should see longer NRTs for Paired+Hit trials and shorter NRTs for CS+Miss trials. To answer this question, we calculated NRTs from neural activity recorded during these trials. However, we find that there is no difference among NRTs regardless of trial types ($\chi^2(2, 1120) = 1.91, p = 0.385$, Kruskal-Wallis Test, Figure 7D). Thus, we can conclude that the timing of reward timing activity is not influenced by ongoing licking activity.

These data indicate that reward timing expression cannot be explained by the initiation or continuation of licking behavior.
2.2.4: *Binocular Reward Timing Responses are Similar when Cues Predict the Same Delay to Reward*

We have shown that neurons in mouse primary visual cortex, following conditioning in the head-fixed preparation, come to reflect the time interval between a visual stimulus and an expected water reward, consistent with our previous findings (Shuler and Bear, 2006; Chubykin et al., 2013). In this task however, unlike previous reward timing tasks, both visual stimuli predict the same reward at the same time (i.e., the same delay to a reward of the same magnitude). In previous studies, visual stimulation of one eye predicted a...
short delay to reward, whereas stimulation of the other eye predicted a long delay to reward. Neurons under these conditions were reported to express reward timing to one, but not both, of the delays (Shuler and Bear, 2006; Chubykin et al., 2013). But what occurs when different inputs to V1 predict the same reward? Will neurons that express reward timing responses to both stimuli express reward timing using the same response form? If so, how similar will the shape of their responses be and the times they are asserted to report? We address these questions here by assessing neurons’ responses to stimuli differing in their eye-of-origin but predicting the same delay to the same reward. Specifically, we quantified similarity in three ways: Form Concordance, Change in Response Shape, and Change in NRT (see 2.1.14: Neural Data Analysis – Similarity Measurements of Reward Timing Responses for definitions).

In general, we find that of the 253 neurons which express reward timing, 157 (62.06%) express reward timing for both conditioned stimuli (examples shown in Figure 8A). As described above, neurons which express reward timing to both cues are defined “binocular reward timing neurons” as opposed to “monocular reward timing neurons” which express reward timing to only one cue. By definition, the responses from monocular reward timing neurons differ from each other, but we sought to investigate the similarity of reward timing responses from the binocular reward timing neuron population. We first asked for all binocular reward timing neurons how consistently their reward time responses are classified as the same type across the conditioned stimuli. We found that the preponderance of binocular reward timing neurons had responses to the conditioned stimuli that were classified as the same form (150/157, 96%, Figure 8B). We then sought to quantify the similarity of the responses of those neurons which express the same reward timing form across conditioned stimuli.
First, we determined in these binocular reward timing neurons how similar their responses were in their neural report of time. Specifically, we found the absolute value of the difference between calculated NRTs (ΔNRT). This distribution of ΔNRT values was compared to a shuffled distribution of values (in which we shuffled only among the same response forms recorded in sessions with the same conditioned interval; see 2.1.14: Neural Data Analysis – Similarity Measurements of Reward Timing Responses). In doing so, we found that the distribution of ΔNRT values for true pairs was significantly smaller than the shuffled distribution (Z = -12.67, p = 8.60 x 10^{-37}, Wilcoxon rank-sum test, Figure 8C). We next asked how similar these responses were in shape; to quantify, we calculated the Euclidean distance between a neuron’s response to Cue 1 and the response to Cue 2. The distribution of these values was compared to a distribution of shuffled Euclidean distances. As with the ΔNRT value, the distribution of true Euclidean distances was significantly smaller than the shuffled distribution (Z = -16.97, p = 1.39 x 10^{-64}, Wilcoxon rank-sum test, Figure 8D). These results indicate that when two stimuli predict the same outcome at the same delay, the preponderance of binocular reward timing neurons in V1 represent the time interval similarly, in form, timing, and shape.
2.2.5: Neurons Stably Express Reward Timing Across Recording Sessions

The results above show that when neurons express reward timing to both cues, the responses are consistent in form, timing, and shape. We then sought to determine how stable reward timing is across recording sessions. To ascertain which units from a given electrode recorded across days may be regarded as arising from the same neuron, we used the previously described J3 statistic (Moran and Katz, 2014). Briefly, this statistic...
uses the waveform shape of recordings across sessions to determine the likelihood that the neuron is the same (see 2.1.13: Neural Data Analysis – Calculation of J3 Statistic for details). With this method, we found that of our 996 recordings, we recorded from 100 putative repeat neurons (examples shown in Figure 9A). From these 100 repeatedly recorded neurons, we can analyze 200 pairs of responses from subsequent days (each neuron has a response to Cue 1 and Cue 2 on both days). Of these 200 responses, we found that 77 responses express reward timing on one or both days (Figure 9B), and of these 77 responses, 45 responses expressed reward timing on both recording days. By using these responses, we were able to determine the stability in form, timing, and shape across recording sessions. We find that the majority of these responses expressed reward timing with the same form (42/45, 93%, Figure 9B). Next, we sought to quantify similarity in timing and shape as we did in the previous section. We found that the ΔNRT of true pairs was significantly smaller than that of a shuffled control (Z = -2.73, p = 6.40 x 10^-3, Wilcoxon rank-sum test, Figure 9C) and we found that the same is true for distributions of Euclidean Distances (Z = -3.61, p = 3.07 x 10^-4, Wilcoxon rank-sum test, Figure 9D). Together these results suggest reward timing is stable in its form, timing, and shape within the same neurons across days.
2.2.6: V1 Reward Timing Reflects Extra Short Conditioned Intervals

Previous reports of reward timing activity have not explored sub-second interval timing activity. Historically, this has been done to avoid conflating potential reward timing responses with visual-evoked responses. However, the existence of long-latency responses to unpaired visual stimuli (Section 2.3.1: Responses to Visual Stimuli in the

Figure 9: Reward timing is stable in form, timing, and shape across recording sessions. (A) Normalized activity from three example neurons deemed to be the same neuron from different recording sessions which express reward timing similarly on day 1 (purple) and day 2 (pink); average waveforms from entire session shown in insets. (B) Bar chart representing, of all CS responses from repeat neurons, the proportion of responses with reward timing on at least one day (top); of those responses with reward timing on at least one day, the proportion of responses which had reward timing on both days; and of those responses, the proportion of responses which were classified as having the same form (“Concordant Responses”). (C - D) Box plots showing differences in the absolute difference of calculated NRT’s (”ΔNRT”, C) and Euclidean distances (D) for “true pairs” (Day 1 vs Day 2 for the same response) and “shuffle pairs” (Day 1 vs Day 2 of responses with same reward timing form and same conditioned interval). Box limits in box plots represent 25th and 75th percentiles, lines correspond to roughly +/-2.7σ. For demonstration purposes, outliers have been removed from plot. *** in panel C: Z = -2.73, p = 6.40 x 10^-3, Wilcoxon rank-sum test. *** in panel D: Z = -3.61, p = 3.07 x 10^-4.
Absence of an Unconditioned Stimulus) prompted us to investigate the ability of V1 reward timing to represent a sub-second conditioned interval.

To do so, we trained a cohort of wildtype mice as described above with the exception that the delay between the visual stimulus offset and water reward was 500ms. Animal behavior showed a use of the visual stimulus as well as behavioral timing consistent with the expected time of reward (Figure 10B-C). Furthermore, we find that V1 neurons express reward timing even with this short delay (Figure 10A) and that the calculated NRTs of this cohort are significantly shorter than those calculated from the sessions with longer delays described above (Figure 10D). For these comparisons, the minimum value for a valid NRT was changed to 250ms to allow for a better description of neurons recorded with a sub-second conditioned interval.

The minimum value for a valid NRT is necessary as neural responses may have at least two components: a visual-evoked response and an interval timing response. In such a scenario, it would be important that the calculated NRT is reflective of this latter component rather than the former. By using a smaller value for the minimum value, we increase the likelihood of having falsely-short NRTs in neurons with long-latency responses. However, as we expect NRTs to be distributed around the conditioned interval, it is necessary that we choose a minimum value that is appropriate for said interval (in this case 250ms for a 500ms conditioned interval) and compare this distribution to those recorded with longer conditioned intervals. Consequentially, we expect that the curves (especially those from Short and Long Delay Sessions) will shift earlier in time. Nevertheless, if this signal is related to the time between the visual stimulus and the reward, the overall pattern will be maintained (i.e., Extra Short < Short < Long) even if the central tendencies shift leftward. Indeed, this is what we observe when we compare across the three conditioned intervals.
Specifically, NRTs recorded from Extra Short sessions are significantly shorter than those recorded from the Short session ($Z = -3.97, p = 7.10 \times 10^{-5}$) and from those recorded from the Long sessions ($Z = -8.04, p = 9.10 \times 10^{-16}$). Additionally, as seen with a minimum NRT of 0.5s, Short and Long sessions are significantly different from each other with a minimum NRT of 0.25s ($Z = -4.75, p = 2.06 \times 10^{-6}$), though their medians are roughly 80% of their respective target times (Median$_{Short} = 0.85$s, Median$_{Long} = 1.24$s).

The results presented thus far indicate that reward timing activity is present in the primary visual cortex of trained, head-fixed mice; that this timing signal corresponds to the conditioned interval; that it is similar across conditioned stimuli in binocular reward timing neurons; and that there is stability in this signal across recording sessions. What remains

(Figure 10D). Specifically, NRTs recorded from Extra Short sessions are significantly shorter than those recorded from the Short session ($Z = -3.97, p = 7.10 \times 10^{-5}$) and from those recorded from the Long sessions ($Z = -8.04, p = 9.10 \times 10^{-16}$). Additionally, as seen with a minimum NRT of 0.5s, Short and Long sessions are significantly different from each other with a minimum NRT of 0.25s ($Z = -4.75, p = 2.06 \times 10^{-6}$), though their medians are roughly 80% of their respective target times (Median$_{Short} = 0.85$s, Median$_{Long} = 1.24$s).

The results presented thus far indicate that reward timing activity is present in the primary visual cortex of trained, head-fixed mice; that this timing signal corresponds to the conditioned interval; that it is similar across conditioned stimuli in binocular reward timing neurons; and that there is stability in this signal across recording sessions. What remains
to be determined is how V1, as a network, can produce such a representation of time in the various forms. This question will be assessed in Chapter 3: Optogenetic Identification and Perturbation of V1 Reward Timing Activity, but prior to this, it is important to understand the extent to which these long-latency responses exist within V1 prior to and following other manners of conditioning.

2.3: V1 Long-Latency Responses in Other Behavioral Tasks

When a visual stimulus is paired with a water reward, neurons in primary visual cortex (V1) express reward timing activity in one of three canonical forms: a sustained increase of activity until the time of reward (SI), a sustained decrease of activity until the time of reward (SD), or a peak of activity around the time of reward (PK). Such responses can be referred to, in general, as "long-latency responses" in that they are prolonged in time following the offset of the visual stimulus. Although these responses are present after reward conditioning and the timing of these responses shift following changes in the conditioned interval (Figure 6 and Figure 10), it is unclear if these responses exist prior to conditioning or if they would reflect conditioned intervals outside of reward conditioning.

In a previous reward timing report, researchers found that there is prolonged neural activity within rat V1 that appears following repeated exposure to visual stimuli in the absence of reward conditioning (Zold and Hussain Shuler, 2015). This response corresponds to the intensity of the visual stimulus and, after extensive training, converges to correspond to the expected reward time. This raises the question as to whether neurons in mouse V1 will also express long-latency responses to unpaired visual stimulation after experience. We investigated this presence and the results are described in 2.3.1: Responses to Visual Stimuli in the Absence of an Unconditioned Stimulus.
We were then curious as to whether non-rewarding outcomes are sufficient to engender cued-interval timing activity within V1. According to the theorized RDE learning rule (Gavornik et al., 2009), a neuron need only access to the conditioned stimulus and the reinforcement signal to produce interval timing activity. Previous work has shown acetylcholine (ACh) released from the basal forebrain (BF) to be a necessary and sufficient reinforcement signal to engender reward timing activity in V1 (Chubykin et al., 2013; Liu et al., 2015). Historically, BF-ACh neurons have been hypothesized to provide reinforcement signals to the cortical mantle (Mesulam et al., 1983; Richardson and DeLong, 1988), and only with recent technological advances has it been possible to understand their response properties to outcomes. For example, through targeted recordings, it has been recently shown that BF-ACh neurons rapidly respond to positive and negative outcomes (Hangya et al., 2015) within the BF and a related study has shown that this reinforcement signal does affect ongoing cortical activity (Guo et al., 2019). As it has been shown that ACh, by itself, is sufficient to engender timing activity within V1 (Liu et al., 2015), we hypothesize that any event capable of activating BF-ACh neurons would be a sufficient outcome to engender timing activity within V1. We test this hypothesis by conditioning monocular visual stimuli with a neutral, binocular visual stimulus (2.3.2: Neutral Conditioning Does Not Engender Interval Timing Activity) or with an aversive tail shock (2.3.3: Aversive Conditioning Does Not Engender Timing Activity). We find that neither are sufficient to engender timing activity within V1.

Finally, we wish to determine whether V1 neurons of mice can correlate with an animal's visually-timed action, as has been shown in recordings of rat V1 (Namboodiri et al., 2015). To this end, we created a novel action timing task wherein the amount of reward received is dependent on the length of time the animal waits to make a lick. We find that, as in rats,
mouse V1 neurons seem to correlate on a trial-by-trial basis with the animal's visually-timed behavior. These data are presented in 2.3.5: Action Timing for the Head-Fixed Mouse.

2.3.1: Responses to Visual Stimuli in the Absence of an Unconditioned Stimulus

To record responses to unpaired visual stimuli, we performed a similar experiment to that described above to record reward timing responses. Specifically, untrained, wildtype animals were presented with monocular visual stimuli with equal probability. However, unlike in the reward timing task, rewards were never given (and these animals were not water deprived nor was a lickport available).

Previous studies suggest the existence of long-latency responses within V1 of untrained rodents (Funayama et al., 2015, 2016; Zold and Hussain Shuler, 2015; Minamisawa et al., 2017) and, as expected we also observe long-latency responses to unpaired visual stimuli. Interestingly, these activity patterns were also able to take the form of the canonical reward timing forms (Figure 11). We recorded 1,082 neural responses from 541 neurons from 10 animals and find that 170 responses to monocular visual stimuli were classified as having long latency responses. These 170 responses were expressed by 121 neurons and the distributions of response forms are as follow: 81 responses expressed by 63 neurons were classified as SI, 66 responses expressed by 50 neurons were classified as SD, and 23 responses expressed by 21 neurons were classified as PK (Figure 11). As with the case of long-latency responses in the reward timing task, we calculated the neural reports of time for these activity patterns recorded in the absence of overt conditioning and find a “pseudo-conditioned distribution” with a median of 0.80 ± 0.52 seconds (Figure 11). Here we use the phrase “pseudo-conditioned” as opposed to native or naïve as these
responses include responses to familiar visual stimuli; familiarity with visual stimuli (in the absence of training) has been shown to influence neural activity within V1 (Gavornik and Bear, 2014; Zold and Hussain Shuler, 2015).

These data indicate that responses to visual cues repetitively delivered can alone result in a distribution of prolonged responses. This observation is consistent with prior work reporting that repeated visual cues gave rise to prolonged V1 responses in rats (Zold and Hussain Shuler, 2015) whose duration initially reflected the intensity of the visual cue, only to subsequently converge to the reward delay time. Additionally, these data further detail the need to define between persistent activity and interval timing activity (as defined at the beginning of this chapter). Specifically, to build confidence that an outcome engenders

Figure 11: Long-latency responses are observed when visual stimuli are unpaired. (A) Normalized activity from three example neurons expressing canonical reward timing forms in response to unpaired visual stimuli: sustained increase (top), sustained decrease (middle), peak (bottom). (B) Proportion of neurons expressing long-latency forms seen during sessions of unpaired visual stimulation. (C) Cumulative probability plot of calculated NRTs from neurons recorded during sessions of unpaired visual stimulation.
interval timing activity, it is necessary to use multiple conditioned intervals to show that activity can reflect and shift based on learned intervals as was shown in reward timing activity (Figure 6 and Figure 10). To increase confidence further, one may wish to change the direction of the shift from Long to Short instead of Short to Long. Results of this experiment will be described in a later section (3.3.2: Alternate Perturbation Strategies do not Influence Network Representation of Time) and are presented in Figure 26.

2.3.2: Neutral Conditioning Does Not Engender Interval Timing Activity

Having demonstrated that visual stimuli paired with delayed water rewards is sufficient to engender long-latency responses within mouse V1 that accord with their delay (“reward timing”), we asked whether other potential outcomes would similarly engender visually-cued timing activity. We began our investigation with a neutral conditioning strategy in which monocular visual stimuli were paired with a delayed binocular visual stimulus (Figure 4 shows a schematic of the neutral conditioning strategy). Briefly, binocular visual stimuli followed monocular visual stimuli at either a short (1 second) or long (1.5 seconds) delay after the offset of a monocular visual stimulus.

Long-latency responses were observed in neutral conditioning (Figure 12). Specifically, we recorded 352 neural responses from 176 neurons from 7 animals and find that 70 responses to monocular visual stimuli were classified as having long-latency responses. These 70 responses were expressed by 51 neurons and the distribution of response forms are as follow: 32 responses expressed by 24 neurons were classified as SI, 35 responses expressed by 26 neurons were classified as SD, and 3 responses expressed by 3 neurons were classified as PK. However, unlike reward timing activity, the central tendencies of the long-latency responses do not reflect the conditioned interval (Short: 0.75 ± 0.39s;
Long: $0.82 \pm 0.82$s). The time course of these long-latency responses were not significantly different across conditioned intervals ($Z = -0.56, p = 0.58$, Wilcoxon rank-sum test, Figure 12).

**Figure 12:** Long-latency responses are observed following neutral conditioning, but do not reflect conditioned intervals. (A) Normalized activity from three example neurons expressing canonical reward timing forms in response to visual stimuli paired with binocular visual stimulation: sustained increase (top), sustained decrease (middle), peak (bottom). (B) Proportion of neurons with long-latency forms seen during sessions of neutral conditioning. (C) Cumulative probability plot of calculated NRTs from neurons recorded during sessions of neutral conditioning when conditioned interval was 1.0s (light green) or 1.5s (dark green). Distributions are not significantly different from one another ($p > 0.05$, Wilcoxon rank-sum test).

### 2.3.3: Aversive Conditioning Does Not Engender Timing Activity

Having demonstrated the existence of long-latency responses to visual stimuli that are either unpaired or paired with delayed binocular visual stimuli, we sought to address whether interval timing (as defined as long-latency responses which reflect conditioned intervals) only occurs when animals learn that visual stimuli are paired with salient outcomes (either rewarding or aversive outcomes).
Given that outcomes of positive and negative valence evoke responses in cholinergic neurons within the basal forebrain (Hangya et al., 2015) and that activation of a subset of these same neurons innervating V1 is necessary and sufficient for V1 to learn reward timing (Chubykin et al., 2013; Liu et al., 2015), it follows that any outcome sufficient to activate BF cholinergic cells could result in interval timing activity. Thus, we hypothesize that conditioning with an aversive US, like conditioning with an appetitive US, would result in similar timing activity. To address this hypothesis, we paired visual stimuli with a delayed electric shock delivered to an animal’s tail.

In a previous report, a tail shock was used as the unconditioned stimulus to train animals on an active avoidance task (Makino and Komiyama, 2015). Specifically, upon receipt of a visual stimulus, mice were required to run on a wheel at a rapid velocity to avoid an aversive, 600µA shock. In our hands, shocks of this magnitude were sufficient to drive neural activity within V1, often to a stronger degree than the visual-evoked response (Figure 13). To minimize the effect of US-evoked activity on recorded neurons, we systematically varied the magnitude of the tail shock while recording extracellularly from V1 neurons. Results of this parameterization experiment are shown in Figure 13 and show an inflection point at 10µA such that shocks greater than this value evoke stronger responses than blank shocks (i.e., magnitude of 0µA). For this reason, we then paired monocular visual stimuli with a delayed, 10µA tail shock to test if aversive outcomes could engender interval timing within V1.
Having decided on an appropriate magnitude to use for aversive conditioning, we then trained animals to associate visual stimuli with a delayed tail shock (as schematized in Figure 4). As before, we observe long-latency responses to visual stimuli after such training. Specifically, we recorded from 1,722 neural responses from 861 neurons from 6 animals and find 79 responses to monocular visual stimuli were classified as having long-latency responses. These 79 responses were expressed by 56 neurons and the distribution of responses forms are as follow: 50 responses expressed by 33 neurons were classified as SI, 21 responses expressed by 18 neurons were classified as SD, and 8 responses expressed by 8 neurons were classified as PK. Similar to neutral conditioning, we find that the central tendencies of NRT distributions do not accord with the conditioned interval (Short: 0.89 ± 0.72s, Long: 0.90 ± 1.26s). The time course of these long-latency
responses were not significantly different across conditioned intervals ($Z = -0.57, p = 0.57$, Wilcoxon rank-sum test, Figure 14).

Figure 14: Long-latency responses are observed following aversive conditioning, but do not reflect conditioned intervals. (A) Normalized activity from three example neurons expressing canonical reward timing forms in response to visual stimuli paired with aversive tail shock: sustained increase (top), sustained decrease (middle), peak (bottom). (B) Proportion of neurons with long-latency forms seen during sessions of aversive conditioning. (C) Cumulative probability plot of calculated NRTs from neurons recorded during sessions of aversive conditioning when conditioned interval was 1.0s (light red) or 1.5s (dark red). Distributions are not significantly different from one another ($p > 0.05$, Wilcoxon rank-sum test).

2.3.4: Comparisons of Timing Activity Across Conditioning Strategies

So far, we have shown that long-latency responses exist within V1 prior to any conditioning. Furthermore, these responses persist through several conditioning strategies: appetitive conditioning where the US is a water reward, pseudo-conditioning where there is no US, neutral conditioning where the US is a second visual stimulus, and aversive conditioning where the US is a 10µA electric shock delivered to the tail. Although these responses are seen across a range of conditioning strategies, only in appetitive conditioning is it observed that the timing of these signals reflect learned conditioned...
intervals. However, a question remains of how similar are these long-latency responses across these various strategies, especially as it relates to the forms observed in the various strategies.

To address this question, we directly compared the proportion of interval timing responses by comparing proportion of response forms among the conditioning strategies to the proportion of response forms to all other. We find that all distributions of timing forms are significantly different from all other forms (all $\chi^2 \geq 9.37$, all $p \leq 2.83 \times 10^{-4}$, chi-square goodness of fit test; Figure 15).

![Figure 15: Different conditioning strategies result in different proportion of timing forms. (A) Probability of being classified as having a timing response and, if so, with what form for all conditioning strategies. (B) Results of multiple chi-squared goodness-of-fit tests comparing probability of responses forms across the various conditioning strategies. All distributions were found to be significantly different from all other distributions.](image)
2.3.5: Action Timing for the Head-Fixed Mouse

So far, we have established that within the primary visual cortex of head-fixed mice there are long-latency responses. These responses exist in the absence of behavioral conditioning (Figure 11) and also exist following various conditioning strategies (Figure 6, Figure 12, and Figure 14). Interestingly, only in the case where visual stimuli are paired with delayed rewards, is it observed that these long-latency responses reflect a learned, conditioned interval (Figure 6 and Figure 10). Previously, reward timing activity has been shown to have a related, but distinct, form of activity referred to as “action timing” (Namboodiri et al., 2015) wherein long-latency responses in rat V1 correlates on a trial-by-trial basis with a visually-guided behavior. Specifically, freely-moving rats were trained to time the initiation of licking after a visual stimulus; the longer the animal waited, the larger the reward the animal received. To determine whether a similar phenomenon is found within mouse V1, we adapted the original action timing task for the head-fixed mice.

In the mouse version of this task, animals received a binocular visual stimulus and then waited to make an initial lick. The longer the animal waited to make this initial lick, the larger the reward the animal received. A distinction between this task and the one used in rats is that following the time of maximum reward availability, there was a slow decline of reward magnitude whereas in the action timing task for rats, there was a sharp decline, as schematized in Figure 16A. Two animals were trained on this novel action timing task and 20 neurons were recorded. As was described in rats, mice are able to delay the initiation of licking activity to maximize the amount of reward delivered (Figure 16B). Within this population, we find that 8/20 (40%) neurons express some long-latency response (Figure 16C). Furthermore, when the neural activity is visualized as a function of time of first lick, we find that there are 6 neurons with activity that appears to precede and correlate, on a trial-by-trial basis, with the time between the visual stimulus and the time
of the first lick (4 example neurons shown in Figure 16D). Though preliminary, these results suggest that, as is the case in rat V1, neurons are able to express timing activity that correlates on a trial-by-trial basis with visually-guided timed actions made by the animal.

![Graph and Table]

**Figure 16: Action timing in mouse primary visual cortex.** (A) Task schematic for action timing in the head-fixed mouse. Animals timed licks off of a binocular visual stimulus (green bar); the longer they waited, the larger volume of water until some maximum (occurring at 1s post-visual stimulus, blue shape). (B) Licking behavior from one example session of action timing. Top: Raster of licking behavior (sorted by first lick time) where animal shows a distribution of first lick times. Bottom: Normalized licking activity across all trials. (C) Table showing number of neurons with a response to the CS, prolonged responses that correlate with licking activity, and neurons that have both a CS response and a response that correlates with licking behavior. (D) Four example neurons which have responses to the CS and responses that seem to correlate with licking activity recorded from one animal across two days of behavior. Top: raster showing spikes (black) and first lick (blue) across all trials of session (sorted by time of first lick). Bottom: Normalized firing activity across all trials. Across all panels, green rectangle represents CS stimulation; vertical, blue, dashed line represents the mean first lick across an entire session.

### 2.4: Summary and Discussion

In the preceding sections, we have shown that reward timing exists within the mouse primary visual cortex in three canonical forms (Figure 6 and Figure 10); that this activity can be consistent and stable (Figure 8 and Figure 9); that these canonical forms are also
observed prior to any conditioning (Figure 11), possibly as a function of stimulus familiarity (Frenkel et al., 2006; Gavornik and Bear, 2014; Cooke et al., 2015; Zold and Hussain Shuler, 2015). Additionally, we report that these forms are also observed in alternate conditioning strategies, although they do not reflect the conditioned interval (Figure 12 and Figure 14); finally, we have shown that neural activity within the primary visual cortex of mice is able to correlate on a trial-by-trial basis with visual-guided actions (Figure 16). In the following sections, I will first describe our interpretation that V1 reward timing activity is reflective of local interactions within V1. I will then contextualize the finding that visual stimuli, in the absence of pairing, result in long-latency responses. I will then discuss the results that conditioning with non-rewarding outcomes is insufficient to engender timing activities. Finally, I will review the results of action timing in the head-fixed mouse and describe its relevance for future experiments.

2.4.1: Reward Timing Activity in the Head-Fixed Mouse is Reflective of Local Interactions

Head-fixed mice were trained to associate a visual stimulus with a delayed reward (Figure 5) and V1 neurons reflected this learned association in one of three forms (Figure 6 and Figure 10). These results replicate previous reward timing reports in the primary visual cortex of freely-moving rats (Shuler and Bear, 2006; Chubykin et al., 2013) and mice (Liu et al., 2015), extend these reports to the head-fixed preparation, and add to reports of non-sensory representations within V1 (Ji and Wilson, 2007; Poort et al., 2015; Fiser et al., 2016; Pakan et al., 2018). As other sensory areas express altered representations following associative learning (McGann, 2015), our understanding of V1 reward timing allows for greater insight into how cortical circuits, generally, can create predictions of future events.
Though we contend that the production of reward timing in V1 is the result of interactions among cells within it, might it be that V1 is reflecting some non-specific global input signal (e.g., arousal or attention)? Our data argue that this alternate explanation is unlikely to be the case. First, a substantial fraction of neurons with reward timing show “cue dominance” (i.e., express reward timing to one, but not both cues; Figure 8). Such specificity in reward timing is difficult to explain if V1 neurons were reflecting some non-specific, global signal. Second, we find that the expression of reward timing is unaffected by how engaged an animal is in our task, as indicated by its licking behavior (a measure known to co-vary with other measures of arousal (Lee and Margolis, 2016); Figure 7). The dissociation between licking and reward timing, then, is not consistent with a global signal being the cause of V1 reward timing activity. Furthermore, previous work shows that lesions, perturbations, and activations specifically directed to V1 influence this timing activity and visually-guided behavior (Chubykin et al., 2013; Liu et al., 2015; Namboodiri et al., 2015). Along with the evidence shown here, these represent several, independent lines of evidence which point to V1 acting as a substrate for learning and producing reward timing activity. That is to say that these results promote the idea that V1 is not merely reflecting patterns of activation from some “smarter” area nor is it that V1 reward timing activity is simply the result of some ongoing global signal. To be clear, however, we are not arguing that V1 is the exclusive site for timing activity. Indeed, much work has shown that timing can be thought of an intrinsic property of cortical circuits (Johnson et al., 2010; Goel and Buonomano, 2016); further, the computational work that has advanced V1 reward timing activity has no circuit elements which are specific to V1 (Gavornik et al., 2009; Huertas et al., 2015). In this way, V1 reward timing activity may be one of many examples of timing activity that occurs throughout the brain.
In sum, the V1 reward timing activity we observe here is in line with previous reports of reward timing activity and furthers the notion that V1 is a substrate for learning and producing reward timing activity. However, what is still unclear is how the forms of V1 reward timing activity are produced. We have shown here that forms can be consistent (Figure 8) and stable (Figure 9) across days. These results do not provide insight as to how V1 produces reward timing in the three canonical forms, but they provide evidence that reward timing activity occurs via strengthening of synaptic weights among neurons within a network (Gavornik et al., 2009). Furthermore, it advances the notion that there may exist some circuit architecture which allows for the production of reward timing in the canonical forms (Huertas et al., 2015). These ideas will be explored in the following chapter where we use a recently described computational model as a hypothesized circuit mechanism that V1 may use to produce reward timing activity in the canonical forms observed.

2.4.2: Long-Latency Responses to Unconditioned Visual Stimuli in V1

In this chapter, I have shown evidence that reward timing activity exists within the primary visual cortex of head-fixed mice and that this timing activity reflects the learned temporal interval where that conditioned interval can be as short as 500ms (Figure 10) and as long as 1500ms (Figure 6) following the visual stimulus offset. However, prior to any conditioning, I have shown that neurons can express long-latency responses of the same form as reward timing responses (Figure 11). These data raise several interesting questions related to latent network architectures and the ability for a network to learn reward timing activity.
Although these long-latency responses are observed in single V1 neurons prior to conditioning, previous work suggests that such persistent responses emerge with familiarity to the stimulus and reflect the stimulus’s intensity (Zold and Hussain Shuler, 2015). Only after reward conditioning do the evoked responses converge toward the expected time of reward. Here we advance our understanding of responses to unconditioned, familiar visual stimuli by showing that the pre-training responses exist within mouse V1 take the same canonical forms of reward timing activity (Figure 11). However, these results have not allowed us to understand how a neural network goes from the native state to a learned state. To address such a question calcium imaging may prove a powerful tool to understanding the evolution of long-latency responses within V1.

Calcium imaging allows for the selective recording of neural activity across the same population of neurons over several days. If we wished to understand whether neurons with long-latency responses prior to conditioning participate within reward timing, then we could gain access to the network’s initial responsiveness and track it as the animal learns that a visual stimulus is predictive of an upcoming reward. However, such a concept and the existence of long-latency responses before training point to a need for more complexity within the computational models that have been used to understand reward timing activity.

In computational instantiations of reward timing activity, it is hypothesized that prior to training, neurons respond transiently to the visual stimulus and activity will decay shortly thereafter (Figure 1 and Figure 2; Gavornik et al., 2009; Huertas et al., 2015). To allow for long-latency responses prior to conditioning, one may wish to adjust the distribution of pre-training synaptic weights within the modeled network to create latent circuit architectures within the untrained network. If this were to be biologically valid, then two related questions are raised: (1) Why do these circuit architectures exist prior to any associative learning? and (2) What is the effect of a learning rule when the circuit architecture already exists?
As it relates to the first question, an over-arching unknown is what neurons are doing outside of reward timing activity. For instance, if a given circuit architecture exists embedded in the complex circuitry (e.g., the core network architecture as proposed by Huertas et al., 2015), then it can be acted on by a learning rule to produce reward timing activity. But to determine why this network may exist in the first place, one would want to know what else these network members respond to. With such information, one could ascertain why these networks exist in the first place and how reward timing may co-opt them to produce behaviorally relevant representations of time.

As it relates to the second question of how the learning rule interacts with pre-existing circuit architectures, the potential pre-existing distribution of long-latency responses within V1 prior to associative learning could require a new understanding of a learning rule’s effects. As it currently stands, the learning rule exists to extend and prolong responses of neurons which (prior to learning) express short-latency responses that relate to the physical properties of the visual world. Following the initial learning, the reinforcement signal can then be used to sculpt the long-latency responses to reflect any new conditioned intervals. In a network where there are already long-latency responses, does the learning rule still create new long-latency responses or does it sculpt existing responses to better approximate a conditioned interval? Studies in which neurons are recorded across days along with computational studies which define possible network adaptations will further our understanding of how biological networks actually change across learning would prove very useful in understanding the transition from naïve to learned within V1.
2.4.3: Non-Reward Conditioning Strategies do not Engender Timing Activity in V1

We have shown that pairing a visual stimulus with a delayed reward results in the representation of time between the visual stimulus and the reward across a range of conditioned intervals (Figure 6 and Figure 10). In the proposed learning rule, for a neuron to produce reward timing activity, it need only access to the conditioned stimulus and the reinforcement signal (Gavornik et al., 2009). This theorized reinforcement signal is thought to be acetylcholine released from the basal forebrain as it is necessary and sufficient for V1 to learn reward timing activity (Chubykin et al., 2013; Liu et al., 2015). BF-ACh neurons have been shown to strongly respond to both rewarding and aversive events (e.g., water rewards, airpuffs, and electric shocks (Hangya et al., 2015; Guo et al., 2019)). As BF or cholinergic activation is sufficient to engender timing activity within V1 (Liu et al., 2015), it follows that if we pair visual stimuli with reinforcers that activate BF-ACh neurons, then we should observe timing activity. We addressed this hypothesis by conditioning visual stimuli with a neutral or aversive outcome and expected that the aversive outcome would engender timing activity. To our surprise, we find that neither strategy engendered visually-cued interval timing activity (Figure 12 and Figure 14). However, we did observe long-latency responses within neurons recorded under these conditioning strategies and the proportion of these responses were different from those observed in reward conditioning or in unpaired visual stimulation (Figure 15). As defined in the beginning of this chapter, these instances of persistent activity do not qualify as “interval timing” as the distributions of neural reports of time do not accord with both conditioned intervals tested. However, it is worth noting that the effect of aversive conditioning is to largely remove persistent activity within V1 (Figure 14) whereas neutral conditioning results in a similar proportion of responses with persistent activity (though with a significantly different distribution of forms) to the proportion of responses with persistent activity observed during pseudo-conditioning (Figure 15). Perhaps it is the case that conditioning with a salient
outcome (e.g., a water reward or tail shock) largely changes the responsiveness of the network (either by engendering reward timing or greatly reducing the amount of persistent activity) whereas less salient conditioning (e.g., neutral conditioning) is insufficient to affect change in the network. Moreover, in the absence of such salient unconditioned stimuli, the network activity could be thought to vary slightly across days allowing for a similar, but different, set of persistent activity patterns. The plausibility of this speculation may benefit by consideration of the relative amount of ACh released by the basal forebrain during conditioning.

As it relates to neutral conditioning, it is possible that pairing a visual stimulus with a second visual stimulus does not engender timing activity because the unconditioned stimulus (i.e., a binocular flash of light) does not adequately activate the BF-ACh system to engender timing activity (though CS responses have been observed in BF cholinergic neurons, the magnitude of the response appears weaker than a US response (Guo et al., 2019)). In absence of ACh as a reinforcement signal, previous work has shown that cortical network activation is sufficient to engender sub-second, timing responses (Johnson et al., 2010; Goel and Buonomano, 2016). Here such a mechanism for engendering timing activity may be ineffective as the conditioned interval is on the order of ones of second and may be beyond the ability for recurrent network activity alone to engender timing activity. Thus, it is expected that these non-salient pairings would not engender timing. Indeed, we find that long-latency forms seen within neutral conditioning do not engender timing activity within recorded V1 neurons. However, we do observe that the distribution of forms is significantly different than the distribution of forms seen in unpaired visual stimulation (Figure 15). It is possible that when visual stimuli are not paired with a salient outcome (either in unpaired stimulation or within neutral conditioning), there is more flexibility within the responses (contrary to the stability seen following reward
conditioning, see Figure 8). Specifically, this flexibility could allow for the network to traverse through any number of neural "states" wherein the proportions of long-latency response forms differ. Such across-day changes outside of conditioning would result in different proportions of long-latency responses. To address such a hypothesized scenario, one could again return to calcium imaging and track the same network of recorded cells to determine if/how a network changes across unpaired visual stimuli.

In contrast to neutral visual stimuli, electric shocks are effective activators of BF cholinergic cells (Hangya et al., 2015; Guo et al., 2019). In aversive conditioning, we then expected that electric shocks would be sufficient to engender timing activity. However, we observe that there is a strong reduction in the proportion of responses with timing activity and that these responses do not reflect the conditioned interval (Figure 14). Such a finding could be understood when we compare what the animal learns in aversive conditioning compared to reward conditioning. In aversive conditioning, the animal is exposed to a delayed shock following a visual stimulus, but there is no behavioral requirement to act on this conditioned interval (in fact, the outcome is inescapable). This lack of a behavioral requirement may make the conditioned interval less behaviorally-relevant for the animal which would thus make it more difficult to express a behavioral or neural representation of time between the CS and US. Indeed, in an active avoidance task wherein a mouse is able to avoid an upcoming electric shock by running fast enough in a delay window, V1 responses are modulated to reflect the relationship of a visual stimulus and an upcoming aversive event (Makino and Komiyama, 2015). In future studies, one could adopt a similar, active-avoidance task wherein the animal avoids an upcoming aversive outcome within a trace interval. However, in such a task care must be taken in interpretations related to the ability for V1 to track the valence of an outcome as it would be confounded with the valence of avoiding the aversive shock.
Another potential reason why interval timing activity is not seen following aversive conditioning may be due to the transient concentration of ACh released relative to its basal concentration. Strong electric shocks strongly activate neurons within V1 (Figure 13). It is possible that these shocks, even at the weakest magnitudes tested, strongly activate BF-ACh neurons within and outside of trials. If ACh is at a high baseline concentration, then evoked ACh upon receipt of an electric shock could be a less effective reinforcement signal in engendering interval timing activity. Furthermore, an electric shock is a strong driver of V1 neural activity which suggests that a shock activates not only the cholinergic system; it is unclear (a) what other responses are evoked by electric shock and (b) what their influence is on the cholinergic signal. Clarity over these two issues may also reveal why aversive conditioning results in the overall decrease of persistent activity within V1. Taken together, it is not clear the relationship between outcomes, evoked responses (cholinergic or otherwise) in V1, and the necessary ACh concentrations for conditioning timing activity within V1. Future studies would benefit from a deeper understanding of how ACh is released following these various conditioning strategies. Recent technological innovations have made such investigations possible. Specifically, cholinergic axons emanating from the basal forebrain have been imaged previously in sensory cortex (Eggermann et al., 2014; Kuchibhotla et al., 2017) and recent innovations have made it possible to directly measure via calcium imaging the levels of ACh within a brain area (Jing et al., 2018).

2.4.4: Action Timing in the Head-Fixed Mouse

Mouse V1 neurons produce reward timing activity in a manner similar to that previously described (Shuler and Bear, 2006; Chubykin et al., 2013). However, in rat V1 it has been shown that V1 neurons can track visual-guided behavior on a trial-by-trial basis
We sought to investigate whether mice can learn such a task and whether neurons may produce timing activity in a similar manner. We find that, indeed, mice can learn a novel action timing task and that neurons seem to have activity patterns which correlate with animal behavior on a trial-by-trial basis (Figure 16). Such an advance allows for us to use the techniques and tools available for the mouse that are not possible in rat to get a deeper understanding of circuit mechanisms underlying this timing activity. Furthermore, such a task allows for a high-throughput behavioral testing of various perturbation strategies (e.g., inactivating V1, lesioning BF-ACh input, etc.). As the current task requires many training sessions for mice to learn (and learning is not guaranteed across mice), future experiments would benefit from a greater parameterization and adaptation of the task as described here.

2.4.5: Concluding Statements

V1 Reward timing activity is seen in the head-fixed mouse in the three canonical forms. In our hands, reward conditioning is the exclusive conditioning strategy that engenders timing activity, but more work is necessary to determine the ability for other outcomes to engender timing activity. Finally, this neural activity seems to be behaviorally relevant as it can correlate, on a trial-by-trial basis, with visually-guided actions. Future computational and biological work will be necessary to better understand how the network transitions from a naïve to learned state; how various unconditioned stimuli influence the release of the presumed reinforcement signal, ACh; and how neural activity within the action timing task is used by the animal. Additionally, results presented in this chapter do not explain how V1 is able to produce reward timing activity with the three canonical forms. In the following chapter we will use a computational model, optogenetic identification of
interneurons, and optogenetic perturbation of the network to understand how V1 produces reward timing with the three canonical forms.
Chapter 3: Optogenetic Identification and Perturbation of V1

Reward Timing Activity

In the previous chapter, I have shown that when mice learn that visual stimuli predict a reward at a fixed delay, neurons in the primary visual cortex (V1) express reward timing activity. This activity represents the time interval between the visual stimulus and the upcoming reward and can take three canonical forms (Figure 6). Although we have a better understanding of how consistent and stable this reward timing activity is, we have yet to gain insight as to how the network produces this activity in these forms.

To gain such insight, we can hypothesize possible circuit architectures that can produce reward timing activity in the forms described. One such circuit motif (as described in 1.2: Reward Timing in Primary Visual Cortex: Learning and Production) posits that V1 reward timing is produced by a theorized network architecture that incorporates both excitatory and inhibitory cells. This network architecture is composed of four populations (three excitatory and one inhibitory) that differ in their relative amounts of recurrent excitation, non-recurrent excitation, and inhibition (as schematized in Figure 17C). This network architecture has two implications: (1) inhibitory interneurons represent time predominantly as the SI form and (2) neurons inhibited by interneurons represent time predominantly as the SD or PK form. In this network, interneurons are treated as one, monolithic group. However, V1 interneurons fall mainly into one of three subpopulations expressing either parvalbumin (PV), somatostatin (SOM), or vasoactive intestinal polypeptide (VIP) (Xu et al., 2010; Tremblay et al., 2016). Each are unique in their connectivity patterns (Pfeffer et al., 2013) and are functionally distinct during stimulus representation in V1 (Atallah et al., 2012; Lee et al., 2012; Wilson et al., 2012). It is unknown if either of the model's implications are borne out in vivo and, if so, how the diversity of interneuron subtypes...
intersects with these implications. In this chapter, I will detail our efforts to test these implications as well as understand what, if any, functional heterogeneity exists within the interneuron subtypes as it pertains to the production of reward timing activity.

Specifically, in 3.2: V1 Produces Reward Timing in a Manner Consistent with a Theorized Network Architecture, I describe our efforts in identifying the various inhibitory interneurons as well as the neurons suppressed by those interneurons to find that V1 produces reward timing activity in a manner consistent with the theorized network architecture. Specifically, we find that PV+ interneurons fulfill aspects of the inhibitory population within the network architecture. These results are built upon in 3.3: Optogenetic Perturbation of V1 Reward Timing as we describe our efforts to perturb network activity and find that only activation of PV+ interneurons influence the network representation of time. Finally, these patterns of reward timing and the differential effects of perturbation is discussed through the lens of known differences and connectivity among these interneuron subtypes in 3.4: Summary and Discussion.

3.1: Materials and Methods

3.1.1: Animal Information and Surgical Procedures

Animals used for the experiments described here expressed channelrhodopsin-2 (ChR2) as described in 2.1.1: Animal Information and Surgical Procedures. Briefly, these animals express ChR2 in one of three inhibitory populations: parvalbumin-positive (PV+), somatostatin-positive (SOM+), or vasoactive-intestinal-polypeptide-positive (VIP+) (as shown in Figure 17B). These animals were implanted with recording microelectrodes that were abutted next to optic fibers (less than 200µm tip-to-tip distance, Figure 17A). This strategy allowed us to optogenetically identify both ChR2-expressing interneurons and
those neurons inhibited (either in a mono- or polysynaptic fashion) by ChR2-expressing interneurons ("Suppressed Neurons").

3.1.2: Neuron Identification – Optogenetic Interneuron Identification

Outside of conditioning, brief (1 or 3ms) laser stimuli were randomly delivered to V1 with an inter-pulse interval randomly drawn from a distribution (between 5 and 10 seconds,
uniformly distributed) while recording from neurons. To identify putative neurons expressing ChR2, we used the latency to the first spike and the probability that a laser evoked a spike. To determine significant latencies to the first spike, we used the calculated $p$-value from the Stimulus Associated Latency Test (SALT). This test has been previously described (Kvitsiani et al., 2013); briefly, this test compares the latencies to a first spike after a laser stimulus to the latencies to a first spike after arbitrary moments in time without a laser presentation. Specifically, a raster of spiking activity is divided into $N$ 10ms bins and the time to a first spike within each bin is recorded. Of the $N$ bins created, one bin is the “test bin” and begins with the laser stimulus onset and one other bin is the “baseline bin” (a bin from the pre-laser time period). For all $N$ bins, a histogram of first-spike latency is created and a modified Jensen-Shannon divergence is calculated between pairs of these distributions. The divergence between the “baseline bin” and all other non-test bins creates a null distribution against which the divergence between the “baseline” and “test” bin is compared. The resultant $p$-value represents the probability that the divergence between the baseline and test bins falls within the null distribution; we have set a conservative alpha of 0.01 as was used in the first description of the method (Kvitsiani et al., 2013). In this way, neurons which have fast and consistent spikes (i.e., fire quickly and with low jitter) after a laser stimulus will be deemed significant. A caveat to this statistical measure occurs when a neuron has a relatively low baseline firing rate. In such a neuron, due to very low firing rates, random, spontaneous activity occurring within the test window could result in a highly-significant $p$-value. For this reason, we also required a neuron to have an action potential in the window immediately following the laser at least 20% of all laser stimulus presentations.
3.1.3: Neuron Identification – Identification of Pyramidal Cells via Spike Width

In addition to interneuron identification we sought to define a population of putative pyramidal cells. We did so by calculating a neuron’s spike width where the spike width is defined as the time difference between when the average waveform first crosses 20% of its peak amplitude and last crosses 20% of its valley amplitude. We then set a threshold at the 75th percentile of non-identified interneurons to define a population of putative pyramidal cells.

3.1.4: Neuron Identification – Optogenetic Identification of Suppressed Neurons

Additionally, we were interested in classifying neurons whose responses were inhibited by activating ChR2-expressing interneurons (“suppressed neurons”). Specifically, we sought to classify those neurons that putatively do not express ChR2 (i.e., did not pass one or both of the thresholds set to define interneurons, see above). To determine this, we also presented 100ms laser pulses after the brief laser presentations (with the same inter-pulse interval parameters). We then compared the distribution of spike counts in the 100ms immediately prior to and during laser stimulation with the Wilcoxon signed-rank test (WSRT). If a significant difference was found, we then compared the total number of spikes between these two windows across all presentations. Significantly inhibited neurons are those neurons which passed the WSRT and had fewer spikes during laser presentation than before laser presentation. Although we cannot resolve the exact nature of this inhibition (either mono- or polysynaptic), we are able to assess whether populations affected by inhibitory subtype activation follow predictions of the computational model and whether they reveal functional specialization of various interneurons. Additionally, we have limited our analysis to only those neurons which are inhibited by interneuron activation as neurons which are activated during this stimulation could be activated for one of at least
two reasons: (1) they become disinhibited upon activation of interneurons or (2) they express ChR2 but do not pass our statistical thresholds to be defined as expressing ChR2.

3.1.5: Neural Data Analysis – Spiking Characteristics of Identified Interneurons

To characterize identified interneurons (see above for identification methods), we calculated the following: the mean latency to first spike after delivery of a visual stimulus, the mean number of spikes evoked within 100ms of visual stimulation, and the session firing rate during the ITI. The first two values are the mean values across trials of the latency to the first spike following visual stimulation onset or the mean number of spikes that 100ms of visual stimulation evoked within identified interneurons (calculated separately for both ipsi- and contralateral stimuli). To calculate the session firing rate within the ITI, we counted all spikes recorded during the ITI and divided that by the total amount of time spent, in seconds, within the ITI for a given session.

3.1.6: Bootstrap Procedures

To determine significant changes in the proportion of neurons expressing reward timing in the various forms, we used bootstrap analyses. Specifically, for a given population of “test” neurons (e.g., interneurons or suppressed neurons), we randomly selected a sample of neurons of the same size (with replacement) from all other neurons recorded from animals of the same genotype. We then determined the expression of reward timing in this subsampled distribution and created a bootstrap distribution by repeating the process 1,000 times. The resultant $p$-values are the probability that values found in the “test” sample would fall in the bootstrap distribution.
3.1.7: Optogenetic Perturbation Task Designs

The task designs for optogenetic perturbation were largely similar to that of the reward timing task described in the previous chapter (2.1.2: Behavioral Task Design – Reward Timing Tasks). In the main perturbation task used in all animals, interneurons were stimulated for 100ms on 25% of all CS trials. This laser stimulation occurred regardless if the animal licked during the delay and occurred 700ms following the visual stimulus offset and ended 700ms prior to the end of the delay window. Additionally, we performed multiple variations of this perturbation task including: a variation in which the duration of the laser stimulation was varied (MultiDuration), a variation in which the number of 5ms pulses within a 55ms stimulation envelope was varied (MultiPulse), and a variation in which the laser was turned on following the offset of the visual stimulus and remained on for 1s following the end of the delay window for a stimulation time of 2.5s, in total (ExtendedLaser). These tasks are schematized in Figure 18. To note, all cohorts experienced the main perturbation task, only PV-ChR2 animals experienced the MultiDuration task, both PV-ChR2 and VIP-ChR2 experienced the MultiPulse task, and only VIP-ChR2 animals experienced the ExtendedLaser task. Finally, with the exception of some recording sessions during the ExtendedLaser task, the delay window was set to 1.5 seconds following visual stimulus offset. Additionally, a cohort of PV-Cre mice which were made to express the anion channelrhodopsin, stGtACR (Mahn et al., 2018), were also trained on the main perturbation task. The difference being that blue light causes stGtACR to pass anions, thus inactivating PV+ interneurons upon stimulation.

3.1.8: Immunohistochemistry

Expression of ChR2 in interneuron subpopulations was verified with immunohistochemistry, as follows. Brain sections containing V1 were selected for
immunohistochemistry. On day 1, the sections were washed three times for ten minutes each (3x10 minutes) with PBS then were blocked in 10% normal goat serum (NGS) in PBS + Triton 0.1% to permeabilize and reduce background binding to antibodies for 1h. Sections were then incubated with two primary antibodies overnight at 4°C. Sections for all animals were incubated with a primary GFP antibody to recognize the eYFP tag of the ChR2 (Chicken polyclonal, 1:2000, Aves Labs (Catalog Number: GFP-1020)) and one primary antibody to recognize one of three interneuron markers: PV (rabbit polyclonal, 1:2000, Swant (Catalog Number: PV27)), SOM (rat monoclonal, 1:800, EMD Millipore (Catalog Number: MAB354)), or VIP (rabbit polyclonal, 1:2000, Immunostar (Catalog Number: 20077)). Sections were then washed 3x10 minutes with PBS, then incubated overnight at 4°C with secondary antibodies: Alexa 488 Goat Anti-Chicken (1:500, Jackson ImmunoResearch (Catalog Number: 103-545-155)) and Alexa 568 Goat anti Rabbit (PV and VIP) or Rat (SOM) (1:500, Jackson ImmunoResearch (Catalog Numbers: 111-065-144 and 112-585-143)). Sections were washed with PBS, mounted on glass slides, and coverslipped with Fluoromount-G mounting medium (Electron Microscopy Sciences). To control for unspecific staining, sections were stained in an identical manner except primary antibodies were omitted. Co-expression was as expected given animal's genotype (Figure 17B).
3.2: V1 Produces Reward Timing in a Manner Consistent with a Theorized Network Architecture

Recent computational work posits that a simple network motif can produce reward timing activity with the three known response forms (Huertas et al., 2015). This network motif is derived from a recurrent network of excitatory cells with broad and sparse inhibition; it contains one population of inhibitory cells and three populations of excitatory cells which differ based on levels of recurrent excitation, non-recurrent excitation, and inhibition (schematized in Figure 17C). Two experimentally tractable implications of this network motif are: (1) inhibitory interneurons should represent reward timing predominantly as the

**Figure 18: Multiple optogenetic perturbation tasks were used to determine downstream effect of interneuron populations.** Four major perturbation tasks were used and are schematized here. In all schematics, the green rectangle represents the visual stimulus, cyan represents laser stimulation (with darker colors representing stronger stimulation), and blue represents water reward. The amount of space taken up within the rectangle represents the probability of an event occurring. (A) Main optogenetic perturbation task is shown where a 100ms laser stimulus was given in the middle of the conditioned interval with 25% probability. (B) MultiDuration and MultiPulse optogenetic perturbation tasks wherein the amount of laser stimulation was varied as either the duration of laser stimulation (B) or in the number of 5ms pulses delivered within a stimulation envelope (C). (D) The extended laser optogenetic perturbation, as the name suggests, had an extended laser stimulation following the visual stimulus that extended past the expected reward time by 1s.
sustained increase form and (2) neurons that are inhibited by interneurons should represent time predominantly as sustained decrease or peak forms. Here we test these predictions using mice which selectively express channelrhodopsin (ChR2) exclusively in one of three major interneuron subtypes: those expressing parvalbumin (PV), those expressing somatostatin (SOM), and those expressing vasoactive intestinal polypeptide (VIP, see 2.1.1: Animal Information and Surgical Procedures). By investigating the ability of each interneuron subtype to fulfill the model’s implications, we are able to determine how known interneuron diversity intersects with the proposed network architecture.

3.2.1: Expression of Reward Timing by Optogenetically Identified PV+ and SOM+ Interneurons

With selective ChR2 expression we are able to optogenetically identify interneurons within our recorded population (see 3.1.2: Neuron Identification – Optogenetic Interneuron Identification for identification details). We identified 35/185 (18.9%) PV+ neurons, 15/361 (4.2%) SOM+ interneurons, and 0/203 (0%) VIP+ interneurons. These proportions match the expected relative distribution given that our recordings were made in infragranular layers (Tremblay et al., 2016). Additionally, a control cohort of animals (not expressing ChR2 in any cell population) resulted in 0/247 (0%) recordings returned as expressing ChR2 from these wildtype animals. While we are primarily interested in the reward timing capability of identified interneurons, we also report general spiking characteristics (i.e., latency to spike after CS presentation, CS-evoked number of spikes, and firing rate within the ITI) of these neurons (Figure 19) akin to recent reports from similarly identified interneurons within primary somatosensory cortex (Yu et al., 2019).
As we optogenetically identified PV+ and SOM+ interneurons, we were then able to ascertain their reward timing capabilities. We first determined their ability to produce representations of time and found that their NRTs shift across conditioned intervals ($Z = -3.61$, $p = 3.12 \times 10^{-4}$, Wilcoxon rank-sum test; Figure 20D). Again, we verified that these representations of time are unlikely to be explained by licking behavior as licking, by itself, had no significant effect on ongoing spiking activity ($p = 0.355$, Wilcoxon signed-rank test; Figure 20C). We find that both PV+ and SOM+ interneurons are significantly more likely to express reward timing activity compared to their non-identified counterparts ($p = 8.10 \times$
Having demonstrated that identified interneurons express reward timing activity, we then asked how reward timing is distributed across forms for the subpopulation of interneurons compared to non-identified counterparts. We found that PV+ interneurons are significantly more likely to represent time as a sustained increase of activity compared to non-identified counterparts ($p = 6.33 \times 10^{-25}$, bootstrap – Figure 20F). We then asked the same of SOM+ interneurons and found that, again, these interneurons are significantly more likely to represent time as a sustained increase of activity compared to non-identified counterparts ($p = 3.61 \times 10^{-4}$, bootstrap – Figure 20H).

3.2.2: Expression of Reward Timing by Other Identified Neurons

Although we did not optogenetically identify excitatory cells, we have identified a subpopulation of putative pyramidal cells using waveform shape. We did so, specifically, by using the spike width of a waveform to define a population of recorded cells as wide-spiking (Barthó et al., 2004). To determine the reward timing expression of putative pyramidal cells, we looked at neurons within the top quartile of the spike width distribution. As expected, we find that these neurons express reward timing in all forms (Figure 21). These data are in accordance with the proposed network architecture which suggests that inhibitory interneurons should represent the time interval as the sustained increase form.
Figure 20: Identified interneurons express reward timing as SI form, consistent with theorized network architecture. (A) Cartoon showing the three types of cells we could possibly record from: ChR2-expressing (ChR2+, green), ChR2-negative which are inhibited by ChR2+ cells (ChR2-, brown), and ChR2-negative that are not inhibited by ChR2+ cells (ChR2-, white). Here we investigated reward timing in neurons expressing ChR2 as indicated with the bold lines around the ChR2+ population. (B) Two example raster plots of spikes recorded during “optotagging”. Spikes are black dots plotted with respect to the time of a 3ms laser stimulus; this activity is recorded from a putative PV+ interneuron (left) and a putative SOM+ interneuron (right) and insets show average waveforms during laser stimulation (cyan) and average waveforms during spontaneous activity (black). (C) Distribution of ΔSpikes all identified interneurons, dashed line represents distribution median. (D) Cumulative distribution plots of NRTs calculated from identified interneurons during short delay sessions (light blue) or long delay sessions (dark blue). Vertical, dashed lines represent time of expected reward and horizontal, dashed line indicates median of the distributions. (E) Example PV+ interneuron expresses reward timing as the Sustained Increase form. Inset in top left shows raster plot of spikes during optogenetic identification and inset in top right shows average laser-evoked waveform and average spontaneous waveform (conventions as in B). (F) Left of vertical dashed line: Bar chart showing bootstrap distribution of proportions from non-identified neurons (gray) or actual proportion of reward timing responses for identified interneurons recorded in PV-ChR2 animals (black). Right of vertical dashed line: Bar chart showing distribution of reward timing forms in bootstrap distributions from non-identified neurons (light colors) or actual proportions in identified interneurons (dark colors). Height of bar is either mean of the bootstrap distribution or actual proportion observed; error bars reflect standard deviation of bootstrap distribution. (G-H) Same as E-F but for identified interneurons recorded in SOM-ChR2 animals. * - p < 0.05, *** - p < 0.001, Wilcoxon rank-sum test (D) or Bootstrap (F, H).
An additional component of the theorized network architecture is that neurons whose spiking is inhibited by inhibitory neurons should express reward timing as either the sustained decrease or peak form. To investigate this prediction, we defined cells as “suppressed” by presenting extended laser stimuli (100ms) and recording responses (see 3.1.4: Neuron Identification – Optogenetic Identification of Suppressed Neurons). Consistent with this prediction, we found that neurons which are inhibited by PV+ activation are significantly more likely to represent the time interval as the sustained decrease form compared to non-suppressed counterparts (p = 1.85 x 10^-4, bootstrap; Figure 22A). However, neurons that are inhibited by SOM+ activation were less likely to express reward timing compared to non-suppressed counterparts (p = 1.61 x 10^-5, bootstrap; Figure 22B) and, contrary to the model’s prediction, were significantly less likely to be classified as sustained decrease or peak compared to non-suppressed counterparts (p = 7.49 x 10^-5 and p = 0.020, respectively, bootstrap; Figure 22B). Additionally, we find
that neurons that are inhibited by VIP+ activation are significantly more likely to express reward timing ($p = 0.018$, bootstrap; Figure 22C) and have a significant enrichment of the sustained increase form compared to non-suppressed counterparts ($p = 0.014$, bootstrap; Figure 22C). Together, these results show that PV+ interneurons fulfill the expectations of the theorized interneuron population and provide evidence in favor of the proposed network architecture wherein the reward timing forms arise due to the connectivity among excitatory and inhibitory neurons within V1. In addition, these results also point to functional distinctions of interneuron subtypes in the production of reward timing activity.

### Figure 22: Neurons inhibited by different interneuron subtypes express reward timing forms in varying proportions.

(A) Top: Cartoon schematizing specific PV-negative neurons of interest (i.e., those inhibited by PV+ interneurons); note that though cartoon shows monosynaptic inhibition, we are unable to address the mono- or polysynaptic nature of observed inhibition. Bottom: Bar chart showing bootstrap distribution of proportions in non-suppressed neurons (light colors) or actual proportion (dark colors) of reward timing responses in suppressed neurons recorded in PV-ChR2 animals. Height of bar is either mean of the bootstrap distribution or actual proportion observed; error bars reflect standard deviation of bootstrap distribution. (B) Same as A but for SOM-negative neurons which are inhibited by SOM+ activation. (C) Same as A but for VIP-negative neurons which are inhibited by VIP+ activation. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, bootstrap.

#### 3.3: Optogenetic Perturbation of V1 Reward Timing

The results above suggest that inhibitory interneurons differentially participate and influence reward timing activity within V1. One specific finding is that although PV+ and SOM+ interneurons both represent the time interval with a specific enrichment of the
sustained increase form (Figure 20), but neurons that are inhibited by SOM+ interneurons do not participate in reward timing (Figure 22). This suggests that functional differences could be more useful when considering downstream network effects of these interneuron populations. To address this, we performed optogenetic perturbation experiments wherein we specifically activated or inactivated interneuron subpopulations to determine what, if any, effect this perturbation has on ongoing reward timing activity.

3.3.1: Activation of PV+, but not SOM+ nor VIP+, Interneurons Shortens the Network Representation of Time in V1

Following reward conditioning, we selectively activated interneuron populations in accordance with their expression of channelrhodopsin-2 (ChR2). Although several perturbation strategies were attempted across animal cohorts, we had one constant perturbation strategy (described above in 3.1.7: Optogenetic Perturbation Task Designs). Briefly, it is the same task design as the reward timing task except that on a subset of CS trials, interneurons are activated for 100ms halfway through the expiration of the delay window (Figure 18).

In this task, we find significant influence of interneuron activation only upon stimulating PV+ interneurons (Figure 23). Specifically, we find that activation of PV+ interneurons during the delay shortens the network representation of time as defined as a shortening of NRTs calculated from perturbation trials compared to NRTs calculated from non-perturbation trials ($D = 0.21$, $p = 0.03$, two-sample Kolgomorov-Smirnov test; Figure 23). When a similar analysis is performed for animals expressing ChR2 in SOM+ interneurons, we observe no significant effect on calculated NRTs across the trial types ($D = 0.36$, $p = 0.37$, two-sample Kolgomorov-Smirnov test, Figure 23). Finally, when we perform this task
in animals which express ChR2 in VIP+ interneurons, we find no significant effect on the
calculated NRTs ($D = 0.25$, $p = 0.63$, two-sample Kolgomorov-Smirnov test, Figure 23).
Furthermore, the significant shortening we see up PV+ activation is maintained when we
limit our analyses to neurons that have NRTs greater than the laser stimulation time ($D = 0.34$, $p = 0.001$, two-sample Kolgomorov-Smirnov test; Figure 23). These results are
consistent with expectations based on the expression of reward timing by identified
interneurons and identified suppressed neurons (Figure 23), discussed in more detail
below (3.4.1 Reward Timing Expression by Identified Neurons Provide Evidence in Favor
of Core Network Architecture).

Although we find that activation of PV+ interneurons significantly shortens the network
representation of time (Figure 23), we do not find that such activation influences the
animal’s behavior (Figure 24). Such a result may be due to the fact that in this task, the
animal has non-visual events off of which to time its licking behavior (e.g., time from lick
bout initiation); potential interpretations are discussed below in 3.4.2 Interpretations of
Optogenetic Perturbation Experiments.
Figure 23: Activation of PV+ interneurons, but not SOM+ nor VIP+ interneurons, shortens the network representation of time. (A) Normalized neural activity from an example neuron recorded in PV-ChR2 animals show a shorter representation of time on trials with laser stimulation (cyan trace and star) compared to trials without (black trace and yellow star). Cumulative probability plots (B) and line graphs (C) of NRTs recorded during perturbation (cyan) and normal (black) trials. Inset in Panel B of PV-ChR2 show distribution of NRTs that are later than laser onset time. NRTs recorded from trials in which PV+ interneurons were activated were significantly shorter than NRTs recorded without PV+ activation. This finding is maintained when analysis is limited to NRTs greater than stimulation onset. (D – F) Same conventions as A – C but for SOM+ interneurons; importantly, activation of SOM+ interneurons does not influence network activity. (G – I) same as A – C but for VIP+ interneurons; activation of VIP+ interneurons does not significantly network activity.
3.3.2: Alternate Perturbation Strategies do not Influence Network Representation of Time

We then sought to determine if network effects could be graded based on the strength of PV+ interneuron activation. Additionally, we asked whether additional perturbation strategies in VIP-ChR2 animals could reveal network effects not seen when a single pulse of activation was used. To this end, we developed three additional perturbation strategies: MultiDuration, MultiPulse, and Extended Laser. These strategies are schematized in Figure 18.

PV-ChR2 animals experienced both MultiDuration and MultiPulse sessions. In the MultiDuration task design, NRTs were not affected by the length of duration envelope (F(3, 27) = 2.58, p = 0.08, one-way ANOVA, Figure 25A). Stimulating ChR2 is an unnatural...
manner to affect neural activity as it is difficult, if not impossible, to fully replicate naturalistic activation patterns (i.e., activating neurons in a manner consistent with ongoing neural activity). However, with the MultiPulse task, we attempted to perturb the network in a manner more similar to neural activity by varying the number of 5ms pulses of laser stimulation within a 100ms stimulation envelope (as schematized in Figure 18). In PV-ChR2 animals, we attempted this early in the conditioned interval or in the middle of the conditioned interval (Figure 25B). Regardless of the time point of the laser stimulation, there was no significant effect on recorded NRTs (Early: F(3, 67) = 0.38, p = 0.76, one-way ANOVA; Middle: F(3, 171) = 0.26, p = 0.85, one-way ANOVA; Figure 25B).

Figure 25: Activation of PV+ interneurons in alternate perturbation tasks does not influence the network representation of time. (A) Top: Schematic for MultiDuration Perturbation task. The overall task structure follows the previously described reward timing task; however, bisecting the interval window is the stimulation envelope. Laser stimulation is turned on for 0, 10, 50, or 100ms with equal probability. Middle: Cumulative probability plots of calculated neural reports of time (NRTs) shown for the various stimulation strengths. Bottom: Plot showing response NRTs (connected by lines) across the range of pulse durations. (B) Same conventions as in (A) but for MultiPulse Perturbation task. In the MultiPulse Perturbation task, the strength of the perturbation is set by the number of 5ms laser pulses within the same stimulation envelope. Left panels show results from “Early” sessions wherein the laser stimulation occurred early in the delay window and Right panels show results from “Middle” sessions wherein the laser stimulation occurred in the middle of the delay window.
VIP-ChR2 animals experienced both MultiPulse sessions (where the stimulation occurred in the middle of the conditioned interval) and Extended Laser sessions. As is the case with the typical perturbation strategy, no significant effect was observed during MultiPulse sessions regardless of the strength of activation (F(3, 47) = 0.46, p = 0.71, one-way ANOVA, Figure 26A).

There are several reasons why the negative results observed during the MultiPulse task may be expected in VIP-ChR2 animals. First, although the MultiPulse strategy was taken to approximate a more naturalistic perturbation strategy, it is a weaker perturbation strategy than the standard perturbation strategy (which was insufficient to affect network representations of time in VIP-ChR2 animals). Additionally, our optogenetic perturbation will likely have only activated a small subset of VIP+ interneurons given that we are stimulating deeper layers of V1 where VIP+ interneurons are relatively low in abundance (Tremblay et al., 2016). As such, we attempted a stronger perturbation strategy to see if in doing so we would affect neural activity. In this strategy, we activated ChR2 for 2.5s following visual stimulus offset on a subset of trials. Even with this prolonged activation, we observed no significant effect on the recorded population of NRTs (Z = -0.31, p = 0.76, Wilcoxon rank-sum test; Figure 26B). Considering that disinhibition has been thought to be a potential mechanism for networks to update following associative learning (Letzkus et al., 2015) and that VIP+ interneurons are thought to predominantly innervate other interneurons (Pi et al., 2013; Tremblay et al., 2016), we conjectured that perhaps VIP+ interneurons play a more integral role in learning reward timing rather than producing reward timing. As such, we attempted to block the ability for a network to learn reward timing by activating VIP+ interneurons while the conditioned interval was adjusted. Specifically, we changed the conditioned interval from 1.5s to 1s after visual stimulus offset and stimulated VIP+ interneurons for 2.5s following visual stimulus offset on Paired
trials (i.e., trials in which the animal received a water reward). We observed no significant
effect on the ability of the network to update NRTs to the changed conditioned interval
when compared to NRTs recorded in pre-perturbation, short-delay reward timing sessions
(Z = 0.07, p = 0.95, Wilcoxon rank-sum test; Figure 26C). In contrast, both distributions of
NRTs recorded when the conditioned interval is 1s following visual stimulus offset were
significantly different from the distribution of NRTs recorded when the conditioned interval
is 1.5s following visual stimulus offset (Short_{NoLaser} vs Long: Z = -2.08, p = 0.04, Wilcoxon
rank-sum test; Short_{Laser} vs Long: Z = -2.26, p = 4.7 x 10^{-3}; Figure 26C).
3.3.3: Inhibition of PV+ Interneurons does not Influence Network Representation of Time

Activation of PV+ interneurons shortens the network representation of time (Figure 23). We then sought to assess whether we could achieve bidirectional control of the representation of time by inhibiting PV+ interneurons. As PV+ activation shortens the network representation of time, we expected that inactivation would lengthen the network
representation of time. To test this hypothesis, we virally expressed stGtACR – an anion channelrhodopsin activated by blue light (Mahn et al., 2018) – conditionally into PV+ interneurons using PV-Cre animals and a virus expressing a Cre-dependent version of stGtACR. Outside of conditioning sessions, we delivered 100ms laser stimulation; in doing so, we were able to functionally confirm expression of stGtACR in our recordings by observing neurons which were both activated and inactivated by laser stimulation (Figure 27A-B). After animals were trained on the reward timing task, we then optogenetically inactivated PV+ interneurons for 100ms in the middle of the conditioned interval (as described above and as schematized in Figure 18A). We find that distributions of calculated NRTs are not significantly different regardless if they are recorded on trials with or without PV+ inactivation (D = 0.10, p = 0.99, Wilcoxon rank-sum test, Figure 27C-D). These results could be due to a number of pitfalls that are common to optogenetic perturbation experiments and are discussed below in 3.4.2 Interpretations of Optogenetic Perturbation Experiments.
3.4: Summary and Discussion

Recent computational work has theorized a manner by which a network of cells can produce reward timing activity in the various forms observed (Huertas et al., 2015). The results of this formal model suggest that the heterogeneity of reward timing forms can be captured by a “core network architecture” where the relative amount of inhibition, recurrent
excitation, and non-recurrent excitation differs according to a simple motif (Figure 17). Here we described our efforts in understanding the validity of the core network architecture. First, we addressed two key implications of this model to determine potential biological validity of the proposed network architecture: 1) inhibitory interneurons should reflect reward timing predominantly as the sustained increase form and 2) neurons inhibited by interneurons should express reward timing predominantly as the sustained decrease or peak forms. Using selective expression of channelrhodopsin-2 (ChR2) in interneuron subpopulations, we are able to define these two populations (interneuron and suppressed) outside of behavioral conditioning and probe the reward timing expression in such populations. Following this, we then investigated the amount of control interneurons had over the network representation of time by optogenetically activating or inactivating interneurons within the reward timing task. A simplifying assumption of the model is that all interneurons behave in a similar manner; however, it is known that there are various different interneuron subpopulations within V1 and that they have been shown to have different functional roles when the network represents sensory information (Atallah et al., 2012; Lee et al., 2012; Wilson et al., 2012; Kvitsiani et al., 2013). By selectively expressing ChR2 in specific interneuron subpopulations, we are able to address the model’s implications and determine how these activity patterns intersect with the various interneuron subtypes.

3.4.1 Reward Timing Expression by Identified Neurons Provide Evidence in Favor of Core Network Architecture

Here we identify putative PV+ and SOM+ interneurons (Figure 20B) and find that PV+ interneurons adhere to the model implications. They produce reward timing predominantly as the sustained increase form (Figure 20E-F), and neurons that they inhibit produce reward timing with an enrichment of the sustained decrease form (Figure 22A). These
results can be contrasted with SOM+ interneurons which, while expressing reward timing predominantly with the sustained increase form (Figure 20G-H), largely do not inhibit neurons which express reward timing (Figure 22B). Finally, the manner by which VIP+ interneurons express reward timing remains unknown, but we have shown that those neurons inhibited by VIP+ activation express reward timing predominantly with the sustained increase form (Figure 22C). These results can be understood when known connectivity is incorporated into the network architecture, as discussed below.

Inhibitory interneurons are known to have distinct connectivity among other interneurons and pyramidal cells (Pfeffer et al., 2013; Tremblay et al., 2016). For example, it is known that VIP+ interneurons predominantly innervate other inhibitory interneurons (Pfeffer et al., 2013; Tremblay et al., 2016). As such, it follows that the neurons we defined as inhibited by VIP+ interneurons express reward timing in a manner similar to PV+ and SOM+ interneurons (i.e., with an enrichment of the sustained increase form, Figure 22C). Additionally, the finding that PV+ and SOM+ interneurons produce reward timing in a similar manner may be surprising as they are thought to perform different functions in stimulus representation (Atallah et al., 2012; Lee et al., 2012; Wilson et al., 2012). However, according to the theorized network architecture, interneurons express reward timing as the sustained increase form because they receive input from the excitatory population of sustained increase neurons (Figure 17C). Cortical interneurons are known to receive convergent input from local excitatory cells (Bock et al., 2011; Fino and Yuste, 2011; Hofer et al., 2011; Packer and Yuste, 2011). Specifically, a population of deep layer pyramidal cells has been shown to target both PV+ and SOM+ interneurons (West et al., 2006). Perhaps the similarity in reward timing expression in PV+ and SOM+ populations arises from similar pyramidal cell input to these interneurons. Additionally, if this input is shared with VIP+ interneurons, it would posit that VIP+ interneurons could also express
reward timing as the sustained increase form. Thus, functional differences would be borne out in downstream neurons (e.g., those neurons whose activity is suppressed by interneurons, Figure 22). Finally, when comparing between PV+ and SOM+ interneurons, PV+ interneurons act in a manner more consistent with the inhibitory population proposed in the core network architecture. This also can be understood when one considers that PV+ interneurons are the most abundant type of interneuron and provide the majority of inhibition to pyramidal cells (Markram et al., 2004; Tremblay et al., 2016). Taken together, we are able to overlay known connectivity patterns with the data shown here to hypothesize amendments to the core network architecture (Figure 28).

![Figure 28: Predictions for core network architecture.](image)

The network architecture adapted from Figure 17 from showing a potential manner by which simulated inhibitory neurons can be divided based on reward timing responses and reported connectivity. The gray line connecting PV+ interneurons is purported to exist given computational work; while not evidenced in the data presented here, it cannot be ruled out. In the current proposed architecture, PV+ interneurons fulfill expectations of the theorized inhibitory population as they produce reward timing with the SI form and inhibit neurons expressing reward timing with the SD form.

### 3.4.2 Interpretations of Optogenetic Perturbation Experiments

Here we describe our efforts to further our understanding of the circuit mechanisms underlying V1 reward timing activity by selectively activating or inactivating given interneuron populations. In doing so, we find that activation of PV+, but not SOM+ nor
VIP+, interneurons shortens the overall representation of time by the network (Figure 23). This finding strengthens our updated network architecture (Figure 28) as PV+ interneurons are thought to innervate other neurons participating in reward timing (although we did not observe effects across any VIP+ activation strategy, see below). How this activation intersects with the various reward timing forms is not known and is an open question that provides an excellent foundation to further investigate reward timing activity (see 4.2 Future Experiments to Understand V1 Interval Timing Activity). It is intriguing, however, that no behavioral effect was observed although neurons were significantly impacted by PV+ activation (Figure 24). In this task the animal need only initiate licking sometime within the delay window to receive a reward at the end of the window. Such a task allows for the animal to use many events to time licking behavior. Although the visual stimulus is a very informative event off of which to create an expectation of time, it is possible that the animal is using other events (e.g., time from lick initiation, time from previous lick bout, etc.) to time its actions. Furthermore, it is possible that the animal uses a mixture of strategies to achieve the task requirements. In such a scenario, influencing one (of possibly many) local clocks as we have done by disrupting V1 reward timing activity would have little, if any, effect on ongoing behavior. To determine the behavioral relevance of PV+ influence on V1 reward timing activity, it is necessary to have a task wherein a visual stimulus is the exclusive event off of which an animal times its actions (e.g., action timing as shown in Figure 16). In rats, it has been shown that activation of V1 neurons influences the animal's behavior (Namboodiri et al., 2015) and with the mouse version of the task, we would be able to investigate, with greater granularity, the interplay between interneurons, neural representations of time, and visually-guided timing behavior.

It is important to note that when activating PV+ interneurons, significant network shortening was only observed when the stimulation occurred for at least 100ms across
many trials. This may be explained by insufficient stimulation parameters in the MultiDuration and MultiPulse task designs. In future studies, it may prove beneficial to greater parameterize stimulation protocols to determine to what degree perturbation can be fine-tuned to adjust the effect on the network representation of time. Additionally, we did not observe any effect on the network representation of time when we inactivated PV+ interneurons. This may also be due to insufficient stimulation parameters as described above, but it also may be the result of inefficient gene transfer of the anion channelrhodopsin, or some other experimental limitation. Future studies may benefit from the introduction of alternative inactivating actuators (e.g., a different version of the anion channelrhodopsin (Messier et al., 2018), a light-activated chloride pump (Han and Boyden, 2007), or a light-activated proton pump (Chow et al., 2010)) via genetic crosses as we performed with ChR2 to ensure consistent expression within and across animals.

As mentioned above, we did not observe any significant effect on the network representation of time regardless of the strength of VIP+ interneurons nor did activation of VIP+ interneurons influence the ability for the network to update its reward timing capabilities when the conditioned interval was changed (Figure 26). Given the connectivity known between VIP+ interneurons and other interneurons (Pfeffer et al., 2013; Pi et al., 2013) and the theorized connectivity between VIP+ interneurons and PV+ interneurons participating in reward timing, we would expect that activating VIP+ interneurons would result in prolonging the network representation of time. Furthermore, given the disinhibitory nature of VIP+ interneurons (Pi et al., 2013) and that disinhibition has been proposed as a learning mechanism within sensory cortex (Letzkus et al., 2011, 2015), we would also expect that activation of VIP+ interneurons could preclude the ability of V1 to update. It is possible that the observed lack of effect are true negative results, but it is also possible that we have insufficiently activated VIP+ interneurons based on our implant
strategy. Specifically, we have targeted our recording electrodes to deep layers and placed optic fibers near the electrode tips (Figure 17). However, it is known that VIP+ interneurons are predominantly found in superficial layers of V1. In future experiments, VIP+ interneurons could be more effectively activated if the distance between the optic fiber and recording electrodes is increased. This would allow the optic fiber to stimulate superficial layers while recording sites are situated in deeper layers. Alternatively, recent technological advances allow for spatial control of where, within a given area, laser stimulation is confined (Pisanello et al., 2017). Such a strategy could also prove useful to address VIP+ interneuron participation within V1 reward timing activity.
Chapter 4: General Discussion and Potential Future Directions

In this thesis, I have described our efforts in understanding the production of interval timing activity within the primary visual cortex. A major component of which is the description of our efforts to determine the circuit mechanisms by which the primary visual cortex produces reward timing activity. Using a combination of in vivo electrophysiology, computational modeling, and optogenetic identification and perturbation of circuit elements, we have found evidence in favor of a core network architecture that produces reward timing activity. However, this core network architecture, as previously described, is not sufficiently complex as PV+, but not SOM+ nor VIP+, interneurons fulfill the proposed role of inhibitory elements within the computational model. In other parts of this thesis, I have shown the ability of V1 to produce long-latency responses under a variety of contexts, some of which seemingly correlates with visually-guided behavior. Together, the work described in this thesis grapples with contemporaneous issues within the field and forms the basis of many future experiments related to timing activity within V1. In this chapter, I will first touch on how V1 reward timing relates to ongoing issues within the neuroscience field (4.1: V1 Reward Timing and Contemporaneous Issues in Neuroscience). Specifically, I will discuss how complex neural activity patterns can be classified (4.1.1: On the Classification of Neural Responses), how to localize sources of input or sources of complex activity pattern (4.1.2: On Local vs. Global Signals), and how these data fit into extant interval timing literature (4.1.3: V1 Reward Timing and Other Interval Timing Representations). Following this, I will propose potential future directions related to both the core network architecture (4.2.1: Further Experimentation of Core Network Architecture) and future experiments related to understanding how a network like V1 can learn interval timing activity (4.2.2: Further Experimentation Related to Learning Interval Timing).
4.1: V1 Reward Timing and Contemporaneous Issues in Neuroscience

4.1.1: On the Classification of Neural Responses

A key feature of V1 reward timing activity is that the representation of time comes in three canonical forms: a sustained increase of activity until the time of reward, a sustained decrease of activity until the time of reward, or a peak of activity around the time of reward (Figure 6). In recent years, it has become apparent that neurons within a given region will respond to task variables with various forms of neural activity. Indeed, this finding will likely continue to be seen as researchers are able to record simultaneously from many neurons. How, then, are researchers to make sense of this heterogeneity among recorded neurons? Some researchers have attempted dimensionality reduction and machine learning algorithms to pull out common classes of response types (e.g., Namboodiri et al., 2019) while others have leveraged dimensionality reduction techniques to contextualize responses at a population level (e.g., Russo et al., 2018). Here is the first decision point researchers must take: should individual neural responses be collapsed together and only the information carried by the population be studied or should individual neurons be treated as unique and we attempt to explain all idiosyncratic response properties? As with most questions, the ideal strategy is likely a mixture of these two extremes and is dependent on the overarching question one is asking. For instance, when studying V1 reward timing activity, we have decided to focus more closely on the unique neural responses rather than collapsing across the population of recorded neurons.

Having decided to define neural responses, one is faced with the next decision point: how to define such neural responses. As described above, researchers have used sophisticated analytical techniques to define the common activity patterns of neurons. However, this strategy is not an ideal solution for V1 reward timing as we know that neural activity within V1 reward timing scales with the conditioned interval (Chubykin et al., 2013;
Zold and Hussain Shuler, 2015; Hussain Shuler, 2016). Such a strategy of dimensionality reduction coupled with machine learning has not been successful in teasing apart classes that are reflective of the temporal interval (data not shown). As such, we have decided to classify neural response forms manually, in a blinded fashion (see 2.1.9: Neural Data Analysis – Interval Timing Classification). In addition, we also rely on human observer-based classification as it is a valuable way to classify complex neural activity (Haddad and Marder, 2018) especially as it relates to neural activity that has various timescales. Although this strategy results in the ability to see timing activity within V1, it raises at least two questions: (1) Are there other timing signals within V1 during the reward timing task? and (2) Are the three canonical forms distinct classes or do they exist on a continuum?

To address these questions, it is useful to consider the proposed core network architecture as it currently exists (Huertas et al., 2015). In this model, the core network architecture was derived after pruning negligible synaptic weights on a trained network of recurrent excitatory cells with broad and sparse inhibition. This data-driven strategy resulted in an architecture which produces reward timing in the three canonical forms, but one can, in theory, engineer a circuit architecture which keeps track of time in non-canonical forms (e.g., with negative peaks, activation patterns with inflection points around the time of reward, etc.). As such, it is possible that other forms of reward timing exist within V1, but by looking for the three canonical forms we have overlooked other, potentially useful response patterns. To uncover such forms, it may be useful to revisit previous decision points to treat neurons as an entire population or to adapt classification methods given advances in analytical techniques. Additionally, future studies could take advantage of several innovative technologies that would allow for greater numbers of simultaneously recorded neurons to determine the existence (and capabilities) of rarer forms of activity within reward timing tasks.
As it relates to how distinct these classes are, it again is beneficial to return to the core network architecture. In this architecture, excitatory cells produce the canonical forms of reward timing activity based on their relative amounts of recurrent excitation, non-recurrent excitation, and inhibition (whereas inhibitory cells only express reward timing as the SI form). We discuss these forms as if they are three distinct clusters, but in actuality, one can consider that these forms exist on a continuum. For instance, the PK population can be thought to exist at the nexus of SI and SD as they have recurrent excitation (as the SI class has) and also have inhibition (as the SD class has). By adjusting the relative amount of excitation/inhibition within this population, the exact manner by which they express reward timing activity could be vastly different. In this way, we can contextualize PK responses as being an admixture of SI and SD responses. Although we treat them as separate classes, these neurons are all interacting with one another to produce timing activity; we use the canonical forms to understand such timing activity as they have been seem many times in previous reports of reward timing (Shuler and Bear, 2006; Chubykin et al., 2013; Liu et al., 2015) as a lens to understand how a network can produce and learn to produce timing activity. However, future studies may leverage more sophisticated population analyses as well as higher-density recording strategies to get a better understanding of how these various forms interact with one another to allow for behaviorally-relevant representations to occur.

4.1.2: On Local vs. Global Signals

Historically, it has been thought that interval timing in the brain is the result of a central pacemaker that broadcasts throughout the brain and is accumulated through an integrating mechanism (Gibbon, 1977; Gibbon et al., 1984). However, it has since been
understood that a more biologically relevant mechanism would be to have distributed networks of local clocks throughout the brain (Buhusi and Meck, 2005). Furthermore, experimental work from cortical slices have shown that these networks (in the absence of global signals) are able to produce representations of time (Johnson et al., 2010; Chubykin et al., 2013; Goel and Buonomano, 2016). Our interpretation of reward timing activity shown here is in line with this notion that the production of V1 reward timing activity is the result of lateral weights among the recurrent network with inhibition (Gavornik et al., 2009; Huertas et al., 2015). However, what are the lines of evidence we use to add evidence to this interpretation and how might other researchers tease apart local vs. global phenomena within their experimental results?

Global signals can be thought of occurring through two manners: (1) as a signal from a higher-order area which broadcasts task representations via feedback to lower-level areas and (2) as some global behavioral signal (e.g., attention or arousal) setting the tonic activity of neurons within an area. Sustained activation within higher-order areas have been described previously (Inagaki et al., 2018, 2019) and it is possible that this activity is inherited by V1 to produce reward timing activity. We find this to be an unlikely explanation based on previous work related to acetylcholine. As described previously, acetylcholine is a necessary and sufficient reinforcement signal for V1 to learn reward timing activity (Chubykin et al., 2013; Liu et al., 2015). Such selective perturbations of V1 provide evidence that it is a substrate of learning and such a concept is strengthened by specific activation of V1 neurons causing alterations of visually-guided behavior (Namboodiri et al., 2015). A substrate of learning, here, means that an area is able to learn and produce behaviorally-relevant representation independent of top-down/bottom-up feedback from other areas. When faced with the dilemma of determining substrates of learning, such selective lesions prove very useful in this determination. However, what these
investigations do not do is to define whether an area is the exclusive substrate. For instance, in the computational instantiations of reward timing activity, there is no aspect that is inherent to V1; in fact, many of the components of the models are found throughout sensory cortex. It could be expected, then, that neurons throughout the brain are able to represent the conditioned interval and that V1 is one of potentially many timekeepers. To determine the extent to which other areas are keeping track of time, researchers may wish to use either widefield imaging protocols where activity from large swaths of the brain may be recorded from (Scott et al., 2018) or to use long recording probes that can simultaneously record from several areas at once (Jun et al., 2017). In this way, one would be able to not only determine potential other time-keeping areas, but also be able to further address what the flow of information may be by selective perturbation of cross-area input.

As it relates to the second question (whether reward timing activity is the result of a global signal), we put forth the interpretation that this is not due to some global signal. Although global signals have been shown to influence neural activity within V1 (Niell and Stryker, 2010; Fu et al., 2015), we think that there are several conciliatory lines of evidence that point against this as the sole reason for V1 reward timing activity. First, we find here (Figure 8) and in previous reports (Shuler and Bear, 2006; Chubykin et al., 2013) that neurons with reward timing activity can express cue-dominance (i.e., reward timing activity to one, but not both, reward-predicting cues). It is difficult to imagine a plausible scenario in which a global signal (such as arousal or attention) affects individual neurons with such specificity. Additionally, one can begin to tease apart global signals with anticipatory behavior. Pupil diameter is often used as a marker for an animal's arousal levels and recent work has shown that licking activity correlates very strongly with the size of the pupil (Lee and Margolis, 2016). Here we show that licking behavior can be dissociated and is insufficient to explain reward timing activity within V1 (Figure 7). Of course, that is
not to say that some global signal may influence the activity of some neurons. Indeed, in
the task described here there are neurons which express reward timing to both cues with
quite similar responses. Additionally, while virtually no neurons in V1 expressed activation
to licking alone, there is a distribution of effects that licking has on ongoing activity (Figure 7). We interpret this as the majority of reward timing activity within V1 is related to the
expected time of reward, but this does not exclude the possibility that some neurons are
responding a global signal. Overall, the strategy of careful characterization of the
phenomenology as well as recording of behavior during the task will allow for other
researchers to gain insight as to how much of a neural signal is related to a global signal
and how much is the result of local interactions.

4.1.3: V1 Reward Timing and Other Interval Timing Representations
The data presented here are in line with previous reports of V1 reward timing (Shuler and
Bear 2006; Chubykin et al. 2013; Liu et al. 2015). However, why are these observed
response patterns classified as "interval timing activity" as opposed to general persistent
activity or long-latency responses. Indeed, persistent activity has been seen in cortical
areas and is attributed to holding information in working memory (e.g., (Romo et al.,
1999)). Such a signal would be useful for a given set of tasks in which an agent needs to
remember an upcoming motor command based on the preceding stimulus (e.g., saccade
left), and such a sustained signal would be useful to the agent within the task. Importantly,
in instances of this working memory signal, the signal is quenched by the motor output
(i.e., returning to baseline following execution of the motor command). In contrast, within
reward timing activity, the timescale of the neuron’s response is presumably determined
by local circuit interactions (see above) as opposed to being quenched by a motor
command (or some other global signal). Additionally, we can more formally differentiate
ongoing persistent activity and interval timing activity by operationally defining candidate interval timing activity patterns as having the following characteristics: (1) a representation of an interval between (at least) two associated stimuli and (2) concomitant changes in the neural representation of the interval when the interval is changed (e.g., changing a short conditioned interval to a long conditioned interval).

It is important to note, however, that such a definition does not necessitate that timing activity take any of the forms observed within V1 reward timing activity. Indeed, other signals have been seen in rodent cortex. For example, in the rodent frontal cortex, it has been reported that neurons exhibit increasing or decreasing ramping profiles of activity during interval timing tasks (Narayanan and Laubach 2009; Parker et al. 2014; Xu et al. 2014). This ramping activity is thought to be indicative of integration within neurons (Simen et al. 2011). Such ramping activity is not obviously present within V1, but it is possible that the signal V1 reward timing neurons provide to such areas would allow for integration within downstream populations. For instance, one can imagine the ramping profile is the result of a downstream neuron listening to a population of V1 reward timing neurons; furthermore, the accuracy of the ramp may be influenced by the distribution of NRTs within the reward timing neurons.

As the ramping activity is not an obvious component of these data, it is unlikely that V1 reward timing neurons act as integrating units in the same way as neurons with ramping profiles of activity. Previous work in cultured cortical neurons show that stimulation of network members is sufficient to engender activity within the network that subtends an interval suggesting that timing activity within cortical networks is an intrinsic property of cortical networks (Johnson et al. 2010; Goel and Buonomano 2016). In this light, it is possible that the theorized network architecture which we have posited (Figure 28) is the
specific form that this intrinsic property takes. As the components of the network architecture (e.g., GABAergic subpopulations and excitatory neurons) exist throughout the cortical mantle, it is possible that this motif could be repeated throughout the brain to produce timing activity when other modalities (e.g., touch or sound) are used.

The notion that time can be distributed throughout the brain (as opposed to time being coded by a single, central clock) has been discussed before (e.g., Buhusi and Meck 2005) and it is possible that V1 reward timing activity is an example of such a distributed network. A related question is how does the brain use this signal? An influential model of timing on the order of tens of seconds is the striatal beat frequency (SBF) model (Matell and Meck 2004) wherein time is encoded by the coincident activation of striatal neurons and a bank of cortical oscillators (each oscillating at various rates). Although we do not investigated the oscillatory nature of the neurons recorded here, oscillations within rat V1 have been shown to track a conditioned interval (Zold and Hussain Shuler 2015; Levy et al. 2017). Future studies could investigate further how the reward timing activity we see in V1 is interpreted by downstream areas, in particular the striatum.

4.2 Future Experiments to Understand V1 Interval Timing Activity

4.2.1: Further Experimentation of Core Network Architecture

Here we have provided evidence that V1 produces reward timing activity in a manner consistent with the theorized core network architecture; additionally, we posit amendments to the core network architecture to address functional heterogeneity among interneuron subpopulations. However, there are concepts put forth by the network architecture that should be considered and implications of the network architecture to be tested.
As mentioned above, the manner by which this network architecture was devised (the training and subsequent pruning of a recurrent network of excitatory with broad and sparse inhibition) does not rule out that there are other circuit architectures that could achieve the same goal. In addition to hand-crafting a solution, one can imagine performing a computational experiment in which the starting connectivity matrices varied across many initial networks. These many networks could then be trained and pruned in the same way to determine whether a different solution can be found, if given a different starting point. In this way, the proposed core network architecture may be one of many hypothesized circuit motifs or it could represent a global solution to which all networks converge regardless of initial connectivity patterns. Additionally, given that we find long-latency responses prior to any conditioning, it suggests that the initial parameters in the computational work is heretofore inadequate in describing the full complexity of how the network produces and learns to produce reward timing activity. Furthermore, alternative network architectures which produce reward timing in the three canonical forms may also posit novel forms of reward timing activity which could be used as a guide for previously recorded neurons. In this way, computational work focused on uncovering other solutions to how V1 can produce reward timing activity would allow us to gain insight into alternative solutions and additional implications of how the cortical network behaves in vivo.

If there are multiple core network architectures that achieve reward timing in the canonical forms, it becomes even more imperative to treat the currently proposed core network architecture as a hypothesized network and test all implications. While this thesis has focused on the production of reward timing by interneurons and comparing the responses with the proposed inhibitory population, there are implications that address the production of reward timing activity by excitatory cells. We have shown that putative excitatory cells produce reward timing in all forms (as defined by their average spike waveform, Figure
as expected from the core network architecture, but the network proposes that these are distinct classes of excitatory cells with differential connectivity within the network. Although there is known heterogeneity in pyramidal cell morphology, projection patterns, differential gene expression, and the like (Morishima et al., 2011; Jiang et al., 2015; Radnikow and Feldmeyer, 2018), there are few defined excitatory subpopulations (relative to interneuron subpopulations (Radnikow and Feldmeyer, 2018)). However, with recent technological advances, one could use a combination of calcium imaging and electrophysiology to define connectivity among neurons with reward timing capabilities. Though an ambitious project, the calcium indicator GCaMP could be expressed in V1 excitatory cells and monitor their reward timing capabilities via fluctuations in the intracellular concentration of calcium. After conditioning, a population of these neurons with and without reward timing could then be recorded electrophysiologically using paired, whole-cell recordings to determine their connectivity among simultaneously recorded neurons (as is often performed in slice physiology (Pfeffer et al., 2013)). In this way, it will be possible to determine differences in connectivity among the different excitatory cells which produce reward timing activity in the various forms.

Finally, the core network architecture posits that the reward timing forms are the result of differential, local connectivity. Consistent with this prediction, there is noted heterogeneity among neocortical pyramidal cell connectivity, especially as it relates to their sources of input (Schubert et al., 2001; Holmgren et al., 2003; Song et al., 2005). Such heterogeneity has been seen as evidence for the existence of functional subnetworks within a cortical area (Yoshimura and Callaway, 2005; Yoshimura et al., 2005; Ko et al., 2011). The model’s prediction can be thought of as a null hypothesis (forms are consequential to differential connectivity); an alternative hypothesis to this, however, is that the forms overlay onto some functional aspect of neurons (e.g., projection targets).
These two hypotheses are not necessarily mutually exclusive and it is possible that future experiments will provide greater insight. A possible starting point would be to investigate the manner by which V1 projection neurons produce reward timing. For a downstream area, the PK response could be very useful as it provides a transient and precise “go” signal that can be used to initiate a motor command. Indeed, there are Cre-driver lines which selectively label certain projection neurons (e.g., Emx2-Cre (Kimura et al., 2005)). Additionally, one can define specificity by targeting viral infections to individual downstream areas (e.g., the striatum) in animals which express Cre recombinase in all pyramidal cells. In either way, one could selectively express ChR2 in a specific population of projection neurons and could use similar techniques as those described here to optogenetically identify those projection neurons within a recorded population. In so doing, one would be able to observe whether there is evidence in favor of a functional role of the reward timing forms. This would then allow us to show the intersection between predictions of the theorized network architecture with known heterogeneity among excitatory cells in much the same way this thesis has investigated the diversity of inhibitory interneurons.

4.2.2: Further Experimentation Related to Learning Interval Timing

Though much of the work in this thesis has been inspired by computational investigations of reward timing activity production, there is still much to uncover regarding how the brain learns to keep track of time between two stimuli. First, it is clear that pairing visual stimuli with outcomes influences the responses to the visual stimuli (Figure 15), but it is only appetitive conditioning that results in timing activity reflective of the conditioned interval. Is it possible that neutral and aversive outcomes could engender timing activity? There are two possible manners in which to test for this. The first is to determine whether the
animal has an understanding between the visual stimulus and the upcoming event. As it relates to aversive conditioning, cue-evoked freezing (i.e., immobility following the presentation of a CS after the animal has learned the CS is predictive of an aversive outcome), is a robust behavior to determine how well an animal has learned the association between the CS and US. However, this is not easily measured in the head-fixed mouse. Researchers have observed “freezing” behavior of licking activity when licks are rewarded on a fixed schedule independent of aversive conditioning (Lovett-Barron et al., 2014). Specifically, in the absence of a CS, the animal spontaneously licks, and upon CS presentation, the animal ceases licking (Lovett-Barron et al., 2014). If one were to adapt this task for the timescales of interest, it would be possible to determine the strength of the association and whether an animal has learned the temporal relationship between the CS and US.

The second way to determine whether outcomes are good candidates for engendering timing activity in a conditioning strategy is to directly image the putative reinforcement signal, basal forebrain acetylcholine. Basal forebrain cholinergic neurons have been shown to respond to salient outcomes (e.g., tail shocks and water rewards) within conditioning strategies (Hangya et al., 2015; Guo et al., 2019). As we expect that ACh release is what mediates V1 learning interval timing (Chubykin et al., 2013; Liu et al., 2015), we could image either activity of cholinergic axons within V1 (Eggermann et al., 2014; Kuchibhotla et al., 2017) or directly image the extracellular concentration of ACh within V1 (Jing et al., 2018). In this way, we could address the ability of events to act as potential unconditioned stimuli to engender timing activity. Additionally, a recent report has shown that across the population of basal forebrain cholinergic neurons, there is sustained activation during a conditioned interval (Guo et al., 2019), but it is unclear how this activity may be conveyed to cortex. Does such sustained activity result in a sustained increase of
ACh concentration and, if so, how might this influence our understanding of ACh as a reinforcement signal? Through the imaging of ACh or cholinergic axons within V1, one could make great strides in understanding how V1 learns interval timing activity.

On the topic of how V1 learns interval timing activity, results presented in this thesis raise, at least, two open avenues of research: (1) what is the role of interneurons in learning interval timing activity? and (2) how does the network traverse from a naïve state to a learned state? Disinhibition of pyramidal cells via inhibition of presynaptic GABAergic interneurons has been postulated as a learning mechanism within sensory cortex (Letzkus et al., 2015). Interneurons within mouse V1 are differentially affected by input from the basal forebrain with VIP+ and L1 interneurons being most strongly activated (Alitto and Dan, 2013) – perhaps mediated via differential expression of ACh receptors in different cortical interneuron subpopulations (Chaudhuri et al., 2005; Coppola and Disney, 2018). Indeed, L1 interneurons which express nicotinic acetylcholine receptors have been shown to play a critical role within mouse auditory cortex during fear conditioning and their action is mediated via their connections to PV+ interneurons (Letzkus et al., 2011). Though we have shown that extended activation of VIP+ interneurons does not influence the ability for the network to update reward timing activity (Figure 26), it is possible that we did not appropriately stimulate these cells as they reside in predominantly upper layers (Tremblay et al., 2016) and the stimulation shown here was largely targeted to deeper layers.

Finally, we have shown that there are long-latency responses within V1 to the visual stimuli used here when they are presented in absence of an outcome (Figure 11). It would be very interesting to determine whether reward timing neurons observed in the fully learned network were created anew through conditioning, if the responses that exist prior to conditioning are adjusted to reflect the conditioned interval, or if neurons were selected
from within this population. To determine this, it would be beneficial to track neural activity across many days. Imaging studies provide such an opportunity either by monitoring the intracellular calcium concentration (e.g., via GCaMP) or optically measuring the membrane voltage (e.g., genetically-encoded voltage indicators, or GEVIs, (Xu et al., 2017)). In doing so it would be possible to track a network throughout learning and determine how the network learns to produce reward timing activity. These biological results could then be incorporated into our computational models to further our understanding.

4.3 Concluding Remarks

In sum, the data presented in this thesis deepen our understanding of how V1 can produce timing activity, particularly visually-evoked activity that subtends a conditioned interval between a visual stimulus and a reward. By thoroughly characterizing this activity and comparing response patterns to those proposed by a computational model, we have gained much insight into the ability of V1 to produce behaviorally-relevant representations. Such an interplay between modeling and biological work was a key component to making sense of the various response patterns of inhibitory interneurons and the neurons that are suppressed by the subpopulations of interneurons. As recording techniques become more sophisticated and computational power continues to grow, it will be of great use to continue allowing these two research strategies to interact with each other to answer a question. In addition, we have shown that V1 responses are able to have long-latency responses in a variety of contexts beyond appetitive conditioning (specifically, in untrained animals, in neutral conditioning, and in aversive conditioning). Further, we have shown evidence that neurons are able to correlate activity with visually-guided actions that the
animal takes. These response patterns show that V1 is capable of many higher-order processing abilities.

The activity shown here in V1 is one example of the brain creating behaviorally-relevant representations of the external world. Many other networks throughout cortex and beyond are tasked with a similar problem of connecting disparate stimuli into a single association. How this challenge is overcome is likely through complex neural activity that requires the coordinated activation across a network of interconnected cells. By studying these activity patterns in V1, we not only deepen our understanding of this specific phenomenology, we also gain access to foundational principles underlying how the brain represents the world around us.
References


Chaudhuri JD, Hiltunen M, Nykänen M, Ylä-Herttuala S, Soininen H, Miettinen R (2005) Localization of m2 muscarinic receptor protein in parvalbumin and calretinin


Coppola JJ, Disney AA (2018) Most calbindin-immunoreactive neurons, but few calretinin-immunoreactive neurons, express the m1 acetylcholine receptor in the middle temporal visual area of the macaque monkey. Brain and Behavior 8:e01071.


Curriculum Vitae

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PhD Candidate
Solomon H Snyder Department of Neuroscience
Johns Hopkins University School of Medicine
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Education
2014 – 2020 Johns Hopkins University School of Medicine
Baltimore, MD, USA
PhD, Neuroscience (expected March 2020)
Project Title: The Production of Interval Timing Activity in the Primary Visual Cortex of Mice
Graduate Adviser: Marshall G Hussain Shuler, PhD

2009 – 2013 Brandeis University
Waltham, MA, USA
B.S., Neuroscience and Biology, Magna cum laude
Senior Thesis: Licking Microstructure Reveals Rapid Attenuation of Neophobia
Research Adviser: Donald B Katz, PhD

Academic Research Experience
2015 – 2020 Hussain Shuler Lab, Johns Hopkins University School of Medicine
PI: Marshall G Hussain Shuler, PhD
Research Activities:
• Combined in vivo electrophysiology, optogenetics, and computational modeling to understand the role of various cell types in the production of cortical representations of time in mouse primary visual cortex.

2013 – 2014 Hussain Shuler Lab, Johns Hopkins University School of Medicine
PI: Marshall G Hussain Shuler, PhD
Research Technologist
Research Activities:
• Used in vivo electrophysiology to understand the influence of valence on the representation of cued interval timing activity in mouse primary visual cortex.
• Utilized viral-mediated gene transfer of channelrhodopsin to uncover the behavioral relevance of timing activity within rat primary visual cortex.

2011 – 2013 Behavior, Learning, and Electrophysiology of Chemosensation Lab, Brandeis University
PI: Donald B Katz, PhD
Undergraduate Research Assistant
Research Activities:
- Investigated the time course of taste learning using fine-scale analysis of licking behavior.

2010 – 2011 Perceptual Development Lab, University of Massachusetts Medical School
PI: Teresa V Mitchell, PhD
Undergraduate Research Assistant
Research Activities:
- Performed eye-tracking and EEG experiments in neurotypical children and children with developmental disorders to understand the influence these disorders have on face perception.

Peer-Reviewed Publications


Pre-Print

Invited Commentary

* - These authors contributed equally

Research Presentations
Oral Presentations
2018 A Circuit Dissection of Reward Timing in Primary Visual Cortex. Optogenetic Approaches to Understanding Neural Circuits and Behavior (Gordon Research Seminar)
2018  *A Circuit Dissection of Reward Timing in Primary Visual Cortex.*
Baltimore Brain Series (National Institute on Drug Abuse)

2017  *The role of inhibition in the representation of time in mouse primary visual cortex.*
Junior Scientist Workshop on Neural Circuits and Behavior (Janelia Research Campus).

**Poster Presentations**
2018  *A Circuit Dissection of Reward Timing in Primary Visual Cortex.*
Society for Neuroscience Annual Meeting.

2018  *A Circuit Dissection of Reward Timing in Primary Visual Cortex.*
Optogenetic Approaches to Understanding Neural Circuits and Behavior (Gordon Research Conference)

2016  *The role of inhibition in cortical representations of cued temporal intervals.*
Greater Baltimore Chapter of the Society for Neuroscience Annual Meeting.

2016  *The role of inhibition in cortical representations of cued temporal intervals.*
Pavlovian Society Annual Meeting.

2013  *A Tongue’s Tale: Licking Microstructure Analysis Reveals Rapid Attenuation of Neophobia.*
Association for Chemoreception Sciences Annual Meeting.

2012  *Licking Microstructure Analysis Reveals Rapid Attenuation of Neophobia.*
Brandeis University Department of Science Summer Research Seminar

2011  *Atypical Eye Scan Paths of Individuals with Down Syndrome during Face Perception Tasks.*
Brandeis University Department of Science Summer Research Seminar

**Honors/Awards**

**Funding**

2016 – 2017  Visual Science Training Program Fellow, Johns Hopkins School of Medicine (T32 EY007143)

2016  Honorable Mention, National Science Foundation Graduate Research Fellowship Program

2015  Honorable Mention, National Science Foundation Graduate Research Fellowship Program

2012  World of Work Summer Research Fellow, Brandeis University

2009 – 2013  Lerman-Neubauer Fellow, Brandeis University

2009 – 2013  Presidential Scholar, Brandeis University
Awards
2019  Next Generation Award, Society for Neuroscience
2018  Goodman Young Scholar Award, Johns Hopkins School of Medicine
2018  Finalist, Johns Hopkins Graduate Student Association Student Group Leader of the Year
2017  Finalist, Johns Hopkins Graduate Student Association Student Group Leader of the Year
2013  Reis and Sowul Family Prize for Undergraduate Neuroscience Research, Brandeis University

Teaching Experience
Training
2017 – 2020  Teaching Academy, Johns Hopkins University
• Three-part program to train individuals to be effective classroom leaders and teachers in an undergraduate setting.
• Training included explicit guidance on: syllabus making, lesson planning, active learning exercises, culturally responsive education, and effective classroom strategies.

Classroom Experience
Instructor on Record
2020  The Brain’s Blueprint: Why Cellular Diversity in the Nervous System Matters, Johns Hopkins University
(Co-Instructor)
• Designed a one-month long course to go over why diversity in the nervous system matters for nervous system function (using Marr’s three levels as a scaffolding)
• Developed lesson plans and led lectures relating to topics such as: how to define a cell type, common circuit mechanisms in the nervous system, and neuron/glia interactions.
• Created, administered, and evaluated student work through mini-quizzes, group presentations, and active participation.

Graduate Teaching Assistant
2018  Neuropharmacology, Johns Hopkins University School of Medicine
(Head Professors: Jay Baraban, MD, PhD and Solomon Snyder, MD)
• Assisted with dissemination of course materials via course website
• Provided external support to students (mixture of junior graduate and senior undergraduate neuroscience students).

2018  Neurobiology, Johns Hopkins University School of Medicine
(Head Professors: Seth Margolis, PhD and Paul Worley, MD)
• Held weekly review sessions to explain and contextualize course materials
• Assisted with dissemination of course materials via course website
• Provided external support to students (junior graduate students in non-neuroscience programs)

**Undergraduate Teaching Assistant**

2011  Introduction to Behavioral Neuroscience, Brandeis University  
     (Head Professor: Donald Katz, PhD)
• Provided external support to students (undergraduates) during office hours and review sessions
• Assisted in grading of multiple choice questions and observed grading of short answer/essay questions

**Mentoring Experience**

2018 - 2019  Nithin Lankipalle, Johns Hopkins undergraduate  
              (currently completing undergraduate studies)
2017 - 2018  Katie McCarren, Johns Hopkins undergraduate  
              (currently completing undergraduate studies)
2016 – 2017  Girija Hariprasad, Johns Hopkins undergraduate  
              (currently Teach for America Fellow)
2015 – 2016  Brenda Dzaringa, Baltimore Polytechnic Institute High School Student  
              (currently Vassar College undergraduate)

**Professional Service**

2018 – 2020  Co-Chair, 2020 Gordon Research Seminar: Optogenetic Approaches to Understanding Neural Circuits and Behavior
2017        Program Representative, Annual Biomedical Research Conference for Minority Students (ABRCMS)
2017 – 2019  Student Member, Departmental Diversity Committee, Johns Hopkins School of Medicine
2016 – 2019  NRSA Application Departmental Guide, Johns Hopkins School of Medicine
2016 – 2019  Departmental Broader Impacts Point of Contact, Johns Hopkins School of Medicine
2016        Program Representative, Annual Meeting of Society for Neuroscience Graduate School Fair
2015        Program Representative, Annual Meeting of Society for Advancement of Chicanos and Native Americans in Science (SACNAS)
2015 – 2019  Member, Departmental Student Recruitment Committee, Johns Hopkins School of Medicine
Graduate Student Representative for Neuroscience Department, Johns Hopkins School of Medicine

Leader, Departmental NSF GRFP Workshop, Johns Hopkins School of Medicine

Member, Departmental Student Invited Speaker Committee, Johns Hopkins School of Medicine

Member, Departmental Happy Hour Committee, Johns Hopkins School of Medicine

Science Outreach and Broader Impacts

Co-President (2016 – 2018) and Committee Leader (2019)

Project Bridge, Baltimore, MD
- Project Bridge is a science outreach and communication organization which strives to educate the public on basic science research occurring in Baltimore City and train scientists to better explain their work to non-scientists.
- As president, managed an annual budget of >$2,500 and volunteer base of >100 individuals
- Created and implemented several outreach events including an all-day neuroscience festival, an after-school program combining art and science, and an interactive science presentation program.

Neuroscience Undergraduate Departmental Representative

Brandeis University, Waltham, MA
- Acted as liaison between undergraduates and the neuroscience department to convey concerns with curricula and discuss the needs of students.
- Created a workshop to teach undergraduates how to find a research lab and how to apply for a position. Materials made for workshop are still utilized as an undergraduate resource.