ABSTRACT

Mediodorsal Thalamus Contributes to the Timing of Instrumental Actions

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Abstract

Interval timing relies on a widely dispersed network of cortical and subcortical brain regions. Despite increasing evidence emphasizing the integrative role of the thalamus in cognition, its contribution to timing behavior is poorly understood. Anatomically connected to brain regions within the canonical timing network such as the prefrontal cortex, amygdala, and striatum, the mediodorsal (MD) nucleus of the thalamus is ideally positioned to support proper timing behavior. In order to investigate the potential role of the MD on interval timing, localized and reversible pharmacological inhibition was done. Mice were trained across multiple timing paradigms in order to assess the generality of MD involvement in timing using an operant 30s peak-interval (PI) procedure as well as a 10s head-fixed (HF) task. Additionally, optical inhibition was conducted during a 30s PI procedure allowing for evaluation of MD contributions to different behavioral stages within a single trial. Lastly, whether the MD provides broad or localized support for cortical regions during timing behavior was investigated through optical inhibition of neurons predominantly innervating the prelimbic (PL) cortex, an area with well-established as important for interval timing behavior.

The experiments conducted here provide substantial evidence for MD involvement in timing durations over multiple seconds, with pharmacological inhibition of the MD lead to significant deficits in the accuracy and precision of timing behavior. Additionally, optogenetic experiments found within trial inactivation to be state specific, with MD inhibition leading to delays in the
transition from one behavior to another dependent on time of optical inhibition. Inhibition of MD projections localized mainly to the PL were unable to produce significant changes to timing behavior, though the effects were qualitatively similar. Together the work here demonstrates the importance of the MD to interval timing behavior and provides evidence that it does so through broad interactions across the prefrontal cortex.
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1. Introduction

The ability to process temporal information is a fundamental component of behavior and cognition. From the ability to time millisecond durations necessary for coordinated motor output and speech to the daily fluctuations governing our circadian rhythms, animals are constantly processing the temporal regularities that exist in their environment. Between these extremes resides Interval timing, which covers durations in the seconds-to-minutes range and is vital for many of the cognitive processes we employ on a daily basis such as learning, working memory, and attention. With interval timing being foundational to so many aspects of cognition, extensive research has been dedicated to understanding its neural mechanisms. Additionally, as all neural processes inevitably unfolding over time, it likely comes as no surprise that the number of associated brain regions is as expansive as the cognitive processes with which it is affiliated. Timing correlates have been observed across the cortical mantle as well as striatum, hippocampus, and cerebellum.

Despite this extensive timing network, humans and animals alike are able to perceive a coherent, unified perception to time. The apparent paradox between anatomical dispersion and perceptual unity seems to necessitate the integration of timing information for decision making and unique behavioral responses. This has spurred many theoretical models of timing that, while unique in their principle neural mechanism for track the passage of time, converge into a singular downstream brain area capable of consolidating the temporal
information. This idea is supported by work in animals and humans implicating cortico-striatal circuits provide such a framework. Within this paradigm, time relevant information from various cortical areas converges within the dorsal striatum and can be further integrated with additional sensory and motor signals. Pharmacological manipulations and neuropathologies that disrupt cortico-striatal interactions such as Parkinson’s disease bolster these theories as they also lead to disruptions in interval timing behavior.

However, less clear is whether and to what degree these cortical signals require assistance in order to maintain a veridical representation of time. Other cognitive processes such as working memory and attentional modulation have been shown to rely on interactions with 'higher-order' thalamic nuclei. In particular, recent work has highlighted the importance of interactions between the prefrontal cortex (PFC) and mediodorsal thalamus (MD) in preserving categorical information over multi-second durations. As the PFC is integral to proper timing behavior, it reasons that the retention of temporal information may benefit from these interactions as well. Currently, empirical work aimed towards understanding the potential contribution of the MD in interval timing is sparse and largely dedicated to shorter durations (i.e. less than 1 second). To the best of our knowledge no studies have been dedicated to systematically testing the role of the MD in interval timing behavior. Therefore, in order to determine the relationship between MD activity and interval timing behavior, pharmacological and optogenetic inactivation of the MD will be undertaken. Two distinct timing tasks were utilized to contrast the effects on interval timing behavior with varying
contextual information. Timing behavior for both tasks was analyzed at the session and individual trial level demonstrating important differences across these levels of analysis. Lastly, whether the interactions between the MD and PFC are localized to the prelimbic cortex (PL), a PFC sub region vital for interval timing, will be investigated.

1.1 Interval timing

While time exists as a continuum, there is substantial evidence to suggest that the brain may process temporal information differently depending on a duration's length. One of the clearest examples for this claim is the mammalian suprachiasmatic nucleus of the hypothalamus' (SCN) role as the central pacemaker for circadian rhythms, which is tightly synchronized to the external twenty-four-hour period through the natural light-dark cycle (Moore, 1983). While less clear cut, multiple lines of evidence suggest further divisions can be made between sub-second and supra-second durations based on behavioral properties such as adherence to scalar variability, response to distractors, and ability to perform ordinal comparisons. Additionally, both human MRI studies and animal in vivo work have shown dissociations in the degree to which various brain areas are engaged during timing of sub- and supra-second durations, independent of differences in task structure. This has led some researchers to propose that timing is controlled through distinct sub-second ‘automatic’ and supra-second ‘cognitive’ timing networks (Lewis & Miall, 2003).
The work presented here will focus on interval timing, which is associated with what has been identified as cognitively controlled timing networks and constitutes continuous durations spanning multiple seconds (i.e. supra-second). Timing within this range is not only highly dependent on the proper functioning of the prefrontal cortex, but also the temporal range associated with cognitive processes related to working memory and attentional control. Thus, linking the mediodorsal thalamus both behaviorally and anatomically, providing the conditions most conducive for understanding its role in interval timing behavior.

1.2 Interval timing behavior at various levels of analysis

Understanding timing behavior can be accomplished at varying levels of granularity. The most established and widely used analysis involves assessing the temporal accuracy and precision through fitting data over multiple trials with a mean response function (Catania, 1970; Roberts, 1981; Yin, Lusk, & Meck, 2017). This level of analysis has been highly influential in the establishment and validation of theoretical models of timing (Gibbon, Church, & Meck, 1984; Killeen & Fetterman, 1988; S. A. Oprisan, Aft, Buhusi, & Buhusi, 2018; Staddon & Higa, 1999) and integral to identifying contributing factors to variation in time perception such as accumulation rate of temporal information, memory of known durations, and motivational thresholds (Church, Miller, Meck, & Gibbon, 1991).

However, averaging data presupposes timing behavior is inherently stable over the course of a session. This implicit assumption is incongruent with the known effects of non-temporal factors such as attention (Meck, 1984) and
motivation (Balci, 2014; Galtress, Marshall, & Kirkpatrick, 2012; Ludvig, Balci, & Spetch, 2011; Ludvig, Conover, & Shizgal, 2007) on behavioral output during a timing task. Additionally, common measures related to timing accuracy and precision, while often correlated, are causally independent (Yin et al., 2017) and susceptible to fluctuation over the course of a training session. The idea of extraneous factors influencing timing behavior is not new, with some of the earliest work in the field demonstrating reward size negatively affecting the duration an animal is willing to withhold responding for a food reward (Beer & Trumble, 1965). However, more recent studies have revealed these effects can occur over the course of an individual session leading to changes in both precision and accuracy. In a PI procedure, motivational changes within a session lead to increases in the time taken to engage in the task, decreases in response rate, decreases in the length of responding, and often produced rightward shifts in peak time with no effects on the stop time of task engagement (Balci, Ludvig, & Brunner, 2010). Interestingly, in a head-fixed paradigm with a 10% sucrose solution reward, animals displayed increases in temporal precision through change in both start and stop times while retaining the same temporal accuracy in later trials (Toda et al., 2017). Therefore, behavioral changes exhibited within sessions may be dependent on task parameters. Yet, analysis at this level still requires data to be averaged.

Individual trials constitute the most elemental level at which interval timing behavior can be assessed. In timing tasks such as the PI procedure, animals demonstrate a canonical low-high-low response profile. Specifically, after the
onset of an external cue signaling the start of a to-be-timed duration, animals will initially withhold engaging in the reward contingent behavior, often in the form of pressing a lever. As the duration since the onset of the cue approaches the trained duration, the animal will begin the instrumental behavior at a relatively constant rate. Finally, as the current trial time surpasses the duration at which reward is expected the animal will stop responding and enter a second quiescent state until the trial ends. Pioneering work in this area was the first to demonstrate the gradual increase in response rate found in session averaged data was the result of temporal variation in transitions between discrete states of non-activity and a nearly constant rate of activity (Church, Meck, & Gibbon, 1994), which is likely to be a general behavioral profile independent of the mode of response (Toda et al., 2017). Interestingly, novel analytical approaches have found that neural activity in cortical areas during timing tasks also demonstrate discrete transitions on individual trials (Latimer, Yates, Meister, Huk, & Pillow, 2015) despite showing ramp-like behavior when averaged (Jazayeri & Shadlen, 2015; Leon & Shadlen, 2003). Though, whether neurons exhibit more ramp or step like activity during individual trials is a point of great contention (Latimer, Yates, Meister, Huk, & Pillow, 2016; Shadlen et al., 2016).

Moreover, the interpretation of how pharmacological manipulations affect timing is influenced by the level at which we assess changes in behavior. One of the most investigated being the speeding up (leftward shift) or slowing down (rightward shift) of timed behavior after acutely increasing or decreasing dopamine levels (Çevik, 2003; Maricq & Church, 1983; Maricq, Roberts, &
The canonical interpretation of these results has been that dopamine levels influence the rate at which temporal information is accumulated. However, investigation of individual trials after administration of drugs that increase cytosolic points to differential modulation of the start and stop time, with start times shifting to the left and stop times being unaffected (Gooch, Wiener, Portugal, & Matell, 2007; Saulsgiver, McClure, & Wynne, 2006; Taylor, Horvitz, & Balsam, 2007). Together, these studies have shifted the field’s understanding of dopamine in timing (Balcı, 2014) and highlights the importance of analyzing timing behavior at multiple levels.

1.3 Interval timing networks

Independent lines of research have uncovered an extensive network of brain regions instrumental to both time perception and timed actions including the cortex (Hinton & Meck, 2004; Merchant, Pérez, Zarco, & Gámez, 2013; Parker, Chen, Kingyon, Cavanagh, & Narayanan, 2014), basal-ganglia (Drew et al., 2007; Gouvea et al., 2015; Matell, Meck, & Nicolelis, 2003; Mello, Soares, & Paton, 2015), hippocampus (Jacobs, Allen, Nguyen, & Fortin, 2013; MacDonald, Lepage, Eden, & Eichenbaum, 2011; Yin & Meck, 2014), and cerebellum (Gooch, Wiener, Wencil, & Coslett, 2010). With such an extensive network, many theories of timing have set out to assess the particular contribution of smaller subnetworks based on their role in other aspects of cognition (Meck, Church, & Olton, 1984) or the degree a subnetwork contributes to a particular phase of timed behavior (Petter, Lusk, Hesslow, & Meck, 2016). A number of investigators
have also looked to partition the broader timing network into ‘core’ and ‘peripheral’ networks. Here, the susceptibility to influence of task factors such as signal modality, decision processes, and response requirements would dictate how critical a particular brain region was to time perception (Meck, Penney, & Pouthas, 2008). Areas like the dorsal striatum that is centrally involved in timing across various timing paradigms in both sub-second and supra-second ranges (Bueti, Walsh, Frith, & Rees, 2008; Chiba, Oshio, & Inase, 2008; Jahanshahi, Jones, Dimberger, & Frith, 2006; Matell & Meck, 2004) would represent components of the ‘core’ network. Cortical areas such as the right pre-SMA, left middle frontal gyrus, and left premotor cortex that only show engagement during reproduction tasks would be deemed part of the ‘peripheral’ network.

Evidence from pharmacological (Buhusi & Meck, 2002; Cheng, MacDonald, & Meck, 2006; Lake & Meck, 2013), lesion (Meck, 2006; Meck, 2006; Narayanan, Horst, & Laubach, 2006), electrophysiological (Narayanan & Laubach, 2006, 2009) studies support the inclusion of cortico-striatal connections from the frontal cortex into the ‘core’ timing network. In fact, theoretical timing models such as the striatal beat frequency (SBF) model posits that oscillatory activity from cortical neurons integrate with reward signals from the substantia nigra pars compacta in order to tack the passage of time (Howe et al., 2017; Matell & Meck, 2004; Matell et al., 2003). The coincident activation of these two inputs leads to plasticity on medium spiny neurons (MSN) in the dorsal striatum (Centonze, Picconi, Gubellini, Bernardi, & Calabresi, 2001). These neurons thus
form a ‘memory’ of the temporal contingency between a stimulus and reward that can be accessed later when the same stimulus duration needs to be timed.

The hippocampus, while integral to time perception, has been proposed to play an appreciably different role. In agreement with its established role generating, maintaining, and retrieving episodic memories (Dickerson & Eichenbaum, 2010) much of the work in the field of time and time perception have found the hippocampus to be associated with temporal memory (Meck, Church, & Matell, 2013). Early information processing theories of timing such as the scalar expectancy theory (SET) of timing positioned the hippocampus as a storage of salient durations (Gibbon, 1977). In short, when presented with stimulus implicating a future reward at a known duration, an animal compares a known duration from the past with a current temporal reading. The memory and current reading are associated with cortico-hippocampal and cortical-striatal networks, respectively. When the difference between the two subnetworks reaches a threshold, the animal makes a response.

While these disparate regions of the brain have been proposed to play important roles in interval timing behavior, how they interact with one another is less clear. Theories such SBF have postulated hippocampal information interacts with cortico-striatal information through three potential pathways: (1) into the striatum directly (2) indirectly through accessory cortical regions (3) interacting with striatal output in downstream regions (Yin & Troger, 2011). In the first two potential pathways the memory information is sent to the cortex via the thalamus.
This positions the thalamus as a potential hub for information transfer between important timing subnetworks.

### 1.4 Neural signatures of interval timing

With cells across multiple brain regions showing temporal activity correlates, an important question arises regarding what characteristics make a cell type capable of being classified as a ‘time cell’. Characteristics of principle cell types within the striatum, cortical, and hippocampus all have led investigators to bestow them with this moniker. Specifically, morphology, activity patterns, and neuroanatomical connectivity, make medium spiny neurons (MSNs), CA1 pyramidal neurons, and Purkinje cells (PCs) especially well-suited for temporal processing in the milliseconds-to-minutes range.

MSNs are the principal cell type within the striatum, the main input region of the BG. They receive extensive glutamatergic inputs from virtually the entire cortical mantle and thalamus, along with projections from dopaminergic neurons in the substantia nigra pars compacta (SNc) (Huerta-Ocampo, Mena-Segovia, & Bolam, 2014). Computational models investigating intra-striatal connectivity show that GABAergic collaterals between MSNs as well as connections with cholinergic interneurons allow for highly synchronized activation of MSN ensembles (Moyer, Halterman, Finkel, & Wolf, 2014), increasing the signal-to-noise ratio, a property necessary for precise and accurate timing.

*In vitro* analysis of synaptic modulation indicates that the plasticity of MSNs is ideally suited for temporal processing. Through optical stimulation of
both glutamatergic and dopaminergic afferents synapsing on a MSN within the striatum it was shown that a precise temporal window exists (0.3–2 s) when dopamine must be present after glutamatergic input in order to promote dendritic spine growth (Yagishita et al., 2014). Another study using in vitro stimulation of olfactory inputs to the striatum found the need for an even tighter temporal window, <1 s, to elicit changes in synaptic strength (Wieland et al., 2015). Moreover, this growth was absent in neighboring spines at which dopamine was not released. It should be noted that these findings are from MSNs in the ventral striatum while most timing related activity occurs in more dorsal areas. Despite this fact, there is currently no evidence to suggest that mechanisms of plasticity would differ between the two areas. This type of plasticity may also help explain the ability of cells to readily rescale as new durations become more salient.

Electrophysiological spike recordings have further demonstrated temporal correlates between timing behavior and MSN activity in rats (Matell et al., 2003; Mello et al., 2015). In recent work, Mello and colleagues show temporal scaling in both dorsal striatal populations as well as individual MSNs. Using a fixed-interval (FI) task, the researchers varied the duration of the FI across five durations (i.e., 12, 24, 36, 48, and 60 s) in blocks of 18–40 trials across a single session. 76 of the 112 cells recorded (68%) maintained their ordinal position within the FI duration with near proportional rescaling between blocks. Possibly of most importance, the MSN activity was unable to be fully explained by behavioral motor output as over 50% of response-associated neurons had significant correlations between press initiation and the relative time of the press within the
Furthermore, evolving population dynamics within the striatum during a modified temporal bisection procedure were sufficient to account for perceptual decisions in animals, and was independent of systematic differences in overt behavior (Gouvea et al., 2015).

The temporal reference frame in hippocampal ‘time cells’ was first identified in the CA1 pyramidal layer using a modified spatial alternation task. Rats were trained to alternate between left and right spatial routes on a figure eight maze and run on a wheel during a delay period in-between alternating lap (Pastalkova, Itskov, Amarasingham, & Buzsaki, 2008). Because wheel running ensured that the rat's spatial location and movement was largely fixed during the delay period, sequential activity could not be explained by place cell activity. Indeed, though these cells shared many of the same physiological properties as ‘place cells’ (e.g., theta phase precession), there was no relationship between the sequential order of cells activated while both traversing the maze and on the running wheel. Importantly, both a delay period and a memory component were necessary for temporal sequence activation. By contrast, control animals simply performing on a running wheel or in a water maze without memory demands did not exhibit the same temporal firing patterns (Pastalkova et al., 2008).

This phenomenon was further characterized using a non-spatial object-odor sequence memory task (MacDonald et al., 2011). For this task, rats had to retain the identity of a specific object in order to guide an odor-based choice that took place at the end of a delay period. Nearly half of the neural population activated at specific moments during the delay. An ensemble view of the neurons
showed peak firing rates at successive times during the delay period, and activity also depended on the object briefly presented before the delay started. Furthermore, when the duration of the delay period was suddenly changed, largely new sequential patterns of activity emerged. Thus, just as place cells ‘remap’ to represent different spatial contexts, these cells ‘retimed’ to represent a different temporal context. To address potential confounds between place and time in these previous tasks, a two-odor sequence task was developed for head-fixed immobilized rats (MacDonald, Carrow, Place, & Eichenbaum, 2013). In this task, CA1 pyramidal neurons still displayed time cell sequences that depended on the identity of the odor to begin a trial showing that time cell activity can be dissociated from general movement through space and path integration (a form of distance coding). Work using treadmills further cemented the role of CA1 pyramidal cells time perception by dissociating the temporal firing activity from path integration (Kraus, Robinson II, White, Eichenbaum, & Hasselmo, 2013).

It has been suggested that the temporal coding properties of CA1 cells in the hippocampus arise from either changing cortical states, strengthening of chain-like connectivity causing sequential activation, or a combination of the two (Eichenbaum, 2014). Computational modeling may provide an additional tool to understand how CA1 cells acquire their ability to time (Hasselmo, 2011; Howard et al., 2014; Y. Wang, Romani, Lustig, Leonardo, & Pastalkova, 2015). For example, one model has shown that the firing rate and phase of ‘time cells’ relative to theta oscillations can approximate physical time on a single trial basis (Itskov, Curto, Pastalkova, & Buzsaki, 2011).
Electrophysiological studies in rodent and non-human primates have shown robust correlations between cortical activity and timing behavior across multiple brain regions. One of the most studied neural correlates of timing is ‘ramping activity’ (Figure 1A). These monotonic increases or decreases in firing rate have been observed within the prefrontal (J. Kim, Ghim, Lee, & Jung, 2013; Rainer, Rao, & Miller, 1999) primary motor (Knudsen, Powers, & Moxon, 2014) and posterior parietal cortex (Jazayeri & Shadlen, 2015; Quintana & Fuster, 1999) during various timing tasks. Along with its seemingly ubiquitous presence within the cortex, key observations further indicate ramping as a viable timing mechanism: (i) ramping activity has been demonstrated to occur across multiple timescales from hundreds of milliseconds to multiple seconds (ii) the rate of change can be adjusted through learning different durations (iii) the rate of change during a reproduction task is dependent on the presented duration. Specifically, neuronal activity in the lateral inferior parietal cortex (LIP), recorded in macaques trained on a temporal reproduction task, found changes in firing rate were inversely proportional to the duration being produced. That is, shorter durations had steeper ramping activity therefore reaching threshold sooner (Jazayeri & Shadlen, 2015).
Figure 1: Distribution of timing correlates and associated activity patterns.

(A) Simulation of ramping activity as observed in cortical networks during timing tasks. Parameterized using values from model of neural integration (P. Simen, Balci, de Souza, Cohen, & Holmes, 2011). Theoretical accumulation of activity (bottom) and distribution of time to threshold (top) (B) Heatmap of simulated activity normalized by max firing rate depicting trajectory dynamics found in the cortex, hippocampus, and striatum. (C) Depiction of pauses in Purkinje cell activity within the cerebellum during 300 millisecond delay (ISI) Pavlovian eye-lid conditioning.

However, pharmacological inactivation of ramping activity within these same regions of the cortex, namely the posterior parietal cortex (PPC), during evidence accumulation tasks has negligible effects on stimulus categorization (Erlich, Brunton, Duan, Hanks, & Brody, 2015). Conversely, decreasing prefrontal
cholinergic concentrations reduced temporal precision without disrupting ramping activity demonstrating a potential dissociation between ramping and timing behavior (Zhang, Jung, Larson, Kim, & Narayanan, 2019). Other regions have demonstrated a more causal relationship, namely, ramping in the frontal orienting field (FOF) was found to be necessary for proper performance possibly implicate ramping activity as a general computational motif within cortical circuits of which timing is localized to a particular cortical region.

Recent evidence suggests that neuronal ramping may not, in fact, be ramping at all, but an artifact of bi-stable neuron activity averaged over multiple trials. Analysis of spiking activity within the LIP during individual trials of a motion discrimination task showed 31 of 40 neurons exhibited ‘stepping’ behavior (Latimer et al., 2015) as opposed to the deterministic gradual increase expected of a truly ramping dynamic (c.f. (Shadlen et al., 2016; Zylberberg & Shadlen, 2016)). These findings parallel human studies which have cast doubt on the role of the contingent negative variance (CNV) – an EEG correlate of ramping activity – in temporal processing due to the poor predictive ability of the CNV and the high temporal accuracy demonstrated even after the resolution of the signal (Kononowicz & van Rijn, 2014). How ramping activity develops also remains unclear (Durstewitz & Deco, 2008; Kononowicz & van Wassenhove, 2016).

Larger scale recordings, containing 55–120 simultaneously recorded neurons, have painted a relatively different picture of spiking activity within the cortex during timing. Bakhurin and colleagues (Bakhurin et al., 2017) found putative projection neurons contained activity patterns in which individual
neurons in the orbital frontal cortex (OFC) displayed sequential activity that tiled a 1.5 s delay period following an olfactory cue. This type of firing is reminiscent of “time cell” activity recorded in other brain regions such as the striatum and hippocampus previously described and provides a parsimonious representation of timing signals across distinct brain regions (Figure 1B).

At the mesoscopic level, oscillatory activity within cortical regions has also been theorized as an underlying mechanism for time perception. As with spiking correlates of timing, neural oscillations are pervasive throughout the brain and implicated in a multitude of cognitive processes such as attention, memory, movement preparation and even consciousness (Ward, 2003). Yet, their computational role in these processes is generally unresolved (Sejnowski & Paulsen, 2006). Nevertheless, researchers have long recognized the potential of rhythmically repeating oscillators to track the passage of time from hundreds of milliseconds to tens of minutes (Miall, 1989; Wolpert & Miall, 1996).

Increases of delta range (~4 Hz) oscillations in the medial prefrontal cortex (mPFC) were shown to negatively impact the temporal precision of rats performing a 12 s fixed-interval task. Pharmacological attenuation of these increases in delta through blockage of D1 dopamine receptor (D1DR) signaling mitigated the associated deficits in timing (Narayanan, Land, Solder, Deisseroth, & DiLeone, 2012). Interestingly, D1DR+ neurons in the prefrontal cortex have strong delta frequency coherence with a subset of neurons exhibiting ramping activity implicating a direct link between microscopic spiking and mesoscopic oscillations during timing (Kim & Narayanan, 2019). Additional evidence supports
the existence of spike phase relationships with the mPFC particularly within the theta frequency (5–10 Hz) (Benchenane et al., 2010; Jones & Wilson, 2005; Sirota et al., 2008). Increases in cortical theta have also been associated with interval timing tasks where sustained increases in cortical theta power occur during the encoding of the standard duration in a temporal comparison task (Gu, Jurkowski, Shi, & Meck, 2016; Gu, van Rijn, & Meck, 2015), though whether coherence between HIPP and mPFC remains high across this entire duration has not been tested.

Lastly, and somewhat distinct from previously mentioned timing signals, the cerebellum exhibits precisely timed pauses in principle neurons. It is worth noting this system and timing signature is distinct as it is most strongly associated with durations in the millisecond range and not traditionally associated with interval timing. However recent theories indicate a potential though mitigated role in timing durations over multiple seconds (Petter et al., 2016).

Through integration of two temporally associated stimuli, PCs suppress their firing rate in anticipation of a beneficial movement. Acting as the sole output of the cerebellar cortex, it is thought that PCs represent the core ‘time cells’ of the cerebellum (Figure 1C). Experimentally, the suppression of PC activity, through optogenetic activation of synapsing interneurons (i.e., basket cells) is sufficient to cause a properly timed eye blink response (Heiney, Wohl, Chettih, Ruffolo, & Medina, 2014). At a network level, information about unconditioned stimulus is conveyed via climbing fibers (CF) and the conditioned stimulus
through parallel fibers (PF). The ‘memory’ of the two times and most importantly the difference between the two is stored in PCs, and can be rescaled to the appropriate duration (Johansson, Jirenhed, Rasmussen, Zucca, & Hesslow, 2014). It is important to note that the duration stored in PCs is capable of rescaling. This is the ability to expand and contract the ‘memorized’ duration, similar to that shown in ‘time cells’ in the striatum.

Neural signatures of time perception appear to be as numerous as the brain areas with which they have been associated. While there is no a priori necessity for parsimony in the neural basis for time perception, the fact that ‘time cell’ type activity is found across the striatum, hippocampus, and cortical regions linked to interval timing is worth important consideration. One outstanding question is how these signals can interact, transferring their temporal information across the expansive timing network. Based on its known role as a relay and integrator of information, the thalamus is a promising area for examination.

1.5 The thalamus and cognition

With an intricate array of cortical, subcortical, and cerebellar connections, the mammalian thalamus is a pivotal hub for relaying as well as integrating neural information. Global models of the thalamus have been difficult to generate due to its structural heterogeneity, composed of over 30 anatomically and functionally distinct nuclei. The traditional understanding of the thalamus is a relay between primary sensory regions, primarily in the visual system and the associated brain regions. However, a dichotomy exists across thalamic nuclei
based on the primary sources of strong ‘driver’ inputs capable of propagating signals across synapses and weaker ‘modulator’ inputs (Lee & Sherman, 2011; Theyel, Llano, & Sherman, 2010; Viaene, Petrof, & Sherman, 2011). Using this dichotomy, identifying the sources of ‘driver’ inputs allows for thalamic nuclei to be broadly classified as either sensory-driven first-order (FO) or cortical-driven higher-order (HO) relays, with FO nuclei relays peripheral and subcortical information to a primary cortical area, while HO nuclei relay information between cortical areas (Sherman & Guillery, 2013). In addition to anatomical divisions, these classes exhibit distinct electrophysiological properties with the HO displaying greater amounts of bursting activity (Ramcharan, Gnadt, & Sherman, 2005), a potential indicator for efficient information transfer between brain regions (Elijah, Samengo, & Montemurro, 2015; Zimmerman & Grace, 2018).

Long viewed as a passive relay, the thalamus is increasingly being acknowledged as playing an active role in the transmission of information to the cortex. This has been shown to occur as early as the modulation of visual information in the lateral geniculate nucleus (LGN), a FO thalamic nucleus, based on where attention is directed (McAlonan, Cavanaugh, & Wurtz, 2008; O’Connor, Fukui, Pinsk, & Kastner, 2002). For HO nuclei, such as the pulvinar and mediodorsal thalamus, extensive ‘modulatory’ inputs from cortical and subcortical regions can influence information transmitted via this trans-thalamic route (Rovo, Ulbert, & Acsady, 2012; Sherman & Guillery, 1996, 2002). In addition to these electrophysiological implications, evidence from behavioral
studies have been increasingly implicated HO thalamic nuclei as contributing heavily to cognitive processes.

With its extensive interactions with prefrontal cortical regions, the mediodorsal thalamus has recently garnered much attention for its potential influence on cognition (Mitchell, 2015; Wolff & Vann, 2019). Lesions of the MD have been associated with a multitude of behavioral deficits such as task acquisition (Wolff, Faugère, Desfosses, Coutureau, & Marchand, 2015) attention (Edelstyn, Mayes, & Ellis, 2014) and working memory (Funahashi, 2013; Watanabe & Funahashi, 2012). Recent work using temporary pharmacogenetic inactivation was able to establish a causal role of the MD in working memory (Parnaudeau et al., 2013; Parnaudeau et al., 2015). Specifically, Parnaudeau and colleagues were able to demonstrate that during task acquisition mice trained on a spatial working memory task increased MD neuronal spiking synchronized with PFC local field potentials. However, with temporary inactivation of the MD, changes in synchronization were recorded in the PFC and these changes correlated with errors in learning.

However, the exact role of the thalamus, let alone of individual HO nuclei, to particular aspects of cognition is undecided. Though the aforementioned work presents strong evidence for the involvement of the MD in working memory, it flies contrary to previous lesion work. Systematic exploration of memory deficits associated with lesions to various thalamic nuclei aggregates found anterior aggregates to be vital for temporal order memory, but not medial aggregates that included MD sub regions (Mitchell & Dalrymple-Alford, 2005). Additionally,
excitotoxic lesions of the MD lead to deficits in learning but not performance of a delayed matching task (Hunt & Aggleton, 1998). Additionally, the rats with MD lesions showed an increase in perseverative behavior when switched to a delay non-matching variant of the task.

Less controversial is the well explored contribution of the thalamus, specifically the MD, in tracking the progression of self-movement. This concept has been formalized in the idea of corollary discharge from cortical regions (Crapse & Sommer, 2008). That is, when an animal interacts within its environment, changes in the location of an external stimuli can be caused by either movement in the stimuli itself or movement by the animal relative to the stimulus. This makes it imperative that the brain has a way of deducing positional changes made by the animal. This is done in the form of corollary discharge (Sommer & Wurtz, 2006, 2008).

However, this signal in its most rudimentary form can be seen as a way of tracking changes over time. Using a double-step task, animals were trained to make two sequential saccades in orthogonal directions (i.e. to the right and then up). In experimental sessions muscimol was infused into the MD. Investigators found the second saccade to deviate from the intended target as if the first saccade movement had not occurred (Sommer & Wurtz, 2002). Therefore, the thalamus, particularly the MD, plays a crucial role in updating the cortex about changes in the environment through internal monitoring.

Together, research has impacted the thalamus in a myriad of cognitive functions, beyond its traditional role as a hub for relaying primary sensory
information. A recurrent theme across these roles, be it working memory, attention, or internal movement monitoring, is that it is most heavily engaged when the task occurs over an extended duration. This makes HO thalamic nuclei such as the MD properly suited to assist in interval timing.

### 1.6 Mediodorsal thalamic nucleus and timing

In order to assess the likelihood that the mediodorsal thalamus contributes to interval timing behavior it is important to establish a connection between the MD and timing at three different levels: anatomical, electrophysiological, and behavioral. First, the MD should have connections with brain regions known to be vital for interval timing behavior. Second, the MD should show activity during periods in which temporal information may be relevant. Lastly, cognitive functions that are tied to interval timing behavior should overlap with aspects of cognition dependent on proper MD functioning. While, it is not necessary to meet all three of these criteria for the MD to still play a role in interval timing, it does bolster the prospect.

Being characterized as a HO thalamic nucleus, the MD receives driver inputs from cortical regions, mainly within the frontal cortex and retains a relatively conserved connectivity pattern in both rodents (Krettek & Price, 1977; Oh et al., 2014) and primates (Giguere & Goldman-Rakic, 1988). In addition to connections with the cortex, the MD receives afferents from multiple subcortical areas related to the limbic system and basal ganglia (Groenewegen, 1988). Importantly, all of the aforementioned systems have multiple lines of evidence
demonstrating their importance to interval timing. The mPFC of the rodent, comprised of the anterior cingulate, infralimbic, and prelimbic cortex display neural correlates of interval timing (Kim, Jung, Byun, Jo, & Jung, 2009; Liu et al., 2014; Tiganj, Jung, Kim, & Howard, 2017; Xu, Zhang, Dan, & Poo, 2014) and manipulations within these regions result in timing deficits. In both aspiration lesion (Dietrich & Allen, 1998) and reversible pharmacological (Buhusi, Reyes, Gathers, Oprisan, & Buhusi, 2018) rodents performing a PI procedure exhibited decreased precision, while accuracy remained intact. Similar effects were found in a temporal bisection task where rats had to decide if a given signal duration was closer to a short or long standard duration. Pharmacological inactivation during the task resulted in a flattening of their psychometric functions indicating lower temporal precision (Kim et al., 2009).

In addition to the anatomical overlap with known components of the interval timing network, electrophysiological activity in the MD provides evidence for involvement in time perception. As few studies have looked directly at MD activity during timing tasks, much of this work is indirect and focuses on activity during delay periods on working memory tasks. For example, recordings in the MD during a oculomotor delay response (ODR) task found over 50% of the cells to be delay-dependent (Funahashi, 2013; Schmitt et al., 2017; Watanabe & Funahashi, 2012). Delay-dependent activity has also been found in regions of the ventral nuclear group (Guo et al., 2017) indicating this type of activity may be a common motif of the thalamus in general. However, the presence of delay-dependent firing in the MD is disputed (Han, Lee, Kim, & Jung, 2013; Miller,
Francoeur, Gibson, & Mair, 2017). Though, the discrepancy could be explained by analytical differences in what constitutes a delay-dependent cell. For example, Han et al., 2013 examined ‘continuous differential delay cells’, only defining delay-dependents cells as those whose activity persisted over the entirety of the delay. This is opposed to the categorization criterion in Schmitt et al., 2017, who demonstrated individual MD cells during a 2-alternative forced choice (2AFC) task showed bouts of high activity that tile the delay-interval at a population level. The activity pattern in the latter is more consistent with ‘time cell’ activity found in other brain regions.

Possibly the most crucial finding was that this delay-dependent activity was independent of task rules, with the authors concluding the role of MD activity is sustaining cortical representations. This is supported by additional work showing optogenetic inactivation diminishes temporally clustered cell activity in the rat mPFC on a delay non-match to sample (DNMS) task, with the greatest effects occurring in cells that fired later (i.e. 10s of seconds) in the delay (Bolkan et al., 2017). Previous work looking at the role of the MD in working memory also found the effects of MD inhibition to be greatest with longer delays and well-trained animals (Parnaudeau et al., 2013).

Behavioral studies directly implicating the MD in timing behavior is sparse, though compelling. Rats with pre-training excitotoxic lesions of the MD showed deficits in temporal precision on a duration differentiation task, while post-training lesions lead to immediate deficits in temporal accuracy that could be overcome with additional training (Yu, Gupta, & Yin, 2010). The durations used during the
task were within the range associated with sub-second motor timing (i.e. 400ms, 800ms, 1600ms), therefore, tangentially implicating the MD in interval timing behavior. More direct evidence from Yu et al., 2010, demonstrated both pre- and post-training lesions have a negative impact on timing a 20s inter-response-time (IRT) task, where rats needed to withhold responding for a minimum of 20s after a previous press to receive a reward. Pharmacological inactivation of the MD, as well as other frontal cortex projecting thalamic neurons, have been found to produce deficits in temporal precision (Wang, Narain, Hosseini, & Jazayeri, 2018) and accuracy (Tanaka, 2006) in sub-second timing tasks.

Anatomical, electrophysiological, and behavioral studies provide evidence suggesting a role of the MD in interval timing. It is highly connected to vital timing networks and exerts influence over neural activity within these regions during delay periods which can influence performance. Lastly, though limited and mostly in the sub-second domain, direct examination of its role in timing through pharmacological inactivation has evidenced the necessity for greater exploration into understanding the potential role of the MD in timing behavior and its inclusion into timing network models.
2. Pharmacological Inactivation of mediodorsal thalamus and interval timing behavior

Our understanding of time perception has greatly benefited from observing how timing behavior changes in response to brain perturbations. Lesion work has played a critical role in identifying the neural circuitry foundational to interval timing, with cortical (Binkofski & Block, 1996; Mangels, Ivry, & Shimizu, 1998; Meck, 2006; Nichelli, Clark, Hollnagel, & Grafman, 1995), striatal (Jones & Jahanshahi, 2015; Meck, 2006), hippocampal (Meck et al., 1984; Yin & Meck, 2014), and cerebellar (Callu, El Massioui, Dutrieux, Brown, & Doyere, 2009; Gooch et al., 2010) lesions all contributing to various deficits in interval timing. These findings have allowed for aspects of theoretical timing models to be mapped onto their anatomical analogs as well as inspiring biologically relevant timing models (S. A. Oprisan, Buhusi, & Buhusi, 2018; P. Simen et al., 2011).

Lesion work does suffer from the drawback of being irreversible. In addition, degeneracy between timing subnetworks provides overlapping functionality. This allows one area to compensate, with training, for the loss of another. Degeneracy between systems can occlude researchers from observing the effects of a lesion over time. The use of reversible pharmacological inhibition provides an alternative to traditional lesion studies. Muscimol, a potent agonist of ionotropic GABA receptors (Johnston, 2014) allows for reversible inactivation and avoids potential compensatory found in lesion work. Reversible inhibition using muscimol have shown the crucial role of the frontal cortex (Buhusi et al., 2018) in time perception. As the mediodorsal thalamus is an essential partner of the
frontal cortex in many aspects of cognition, the current chapter is dedicated to understanding its potential role in interval timing through pharmacological inhibition.

**2.1. Materials and Methods**

**2.1.1 Animals**

Data was collected from adult wild type (C57BL/6) mice (n = 10). In all behavioral experiments, mice were maintained on a 12:12 light cycle. During training and testing of the 30s peak interval procedure animals were food deprived, receiving 2.0 - 2.5 grams of rodent chow in addition to 20mg food pellets during testing. During food deprivation animal’s weights were monitored daily to ensure they remained above 85% of baseline. During training and testing of the 10s head-fixed timing task animals were water deprived, receiving 2hrs of free access to water in addition to the 10% sucrose solution received during the task. All experimental procedures were approved by the Duke University Institutional Animal Care and Use Committee.

**2.1.2 Surgery**

Mice were anesthetized with 1.0 to 2.0% isoflurane mixed with 1.0 L/min of oxygen for surgical procedures and placed into a stereotactic frame (David Kopf Instruments, Tujunga, CA). Adult C57BL/6 (n = 10) mice were implanted, bilaterally, with 7mm, 26-gauge guide cannulas over the MD (AP: -1.35 mm, ML: ± 1.48 mm, DV: -3.0 mm, from skull surface at 20° from vertical). Guide cannula coordinates were chosen accounting for infusion cannulas extending out an extra
0.5 mm. Guide cannulas were secured in place with dental acrylic adhered to skull screws. A head post was then cemented to the back of the skull to allow the mice to be head-fixed during experimentation. Mice were singly housed during recovery for at least two weeks before training began.

### 2.1.3 Peak interval procedure

Training for the peak interval procedure is broken into three stages: lever-press training, fixed-interval (FI) training, and peak-interval (PI) training. Lever-press training consisted of the extension of a single lever, either to the right or left of a food port. A food pellet reward (Bio-Serv 20 mg Dustless Precision Pellet) was delivered for each lever press (FR-1). Every 5th press leads to an alternation of which lever was extended. Sessions ended after either successfully receiving 40 rewards or 30 min. Training was considered complete after an animal received 40 rewards prior to the 30 min time limit.

FI training sessions began with the extension of a single lever to either the left or right of the food port, counterbalanced across mice, as well as the illumination of a house light. Individual trials were demarcated by the presence of an auditory cue (white noise; 68 dB). An animal was rewarded for the first lever press occurring after the cue had been on for 30 s. At this time a food reward was delivered, the cue was turned off, and the session entered a variable inter-trial interval ranging from 90 s to 180 s randomly selected from a uniform distribution of values in 5 s increments. If there was no press within 8 s of the 30 s criterion, the trial would end as stated above, but without a reward. There was no penalty for presses occurring prior to 30 s. The development of a “scalloped”
profile for session averaged lever pressing indicated the acquisition of the to-be-learned interval and animals were transitioned to the final stage of training. Each session lasted 2 hrs.

PI training sessions consisted of two trial types: The aforementioned FI trials, and unrewarded probe trials (Figure 2A-B). During probe trials, the house light was turned on for a minimum of 3× longer than the target duration (90 s) plus an additional random amount of time with a mean of 20 s and a Gaussian distribution. Each trial was randomly selected, with a 40% chance of being a probe trial. Each session lasted for 2 ½ hrs.

Figure 2: Experimental setup and behavioral response during PI procedure.

(A) PI procedure set up. One side of an operant box contained a house light, lever and food port (top). Individual trials were demarcated by an auditory cue signaling the beginning of either a rewarded FI or unrewarded PI trial. Well trained animals developed temporally precise presses (slashes) centered around
the expected time of reward. (B) Idealized distribution of pressing activity during PI trials averaged over a session. Peak time and spread of response distribution were used to assess the accuracy and precision of timing ability, respectively.

2.1.4 Head-Fixed timing task

Training for the head-fixed timing task took part in two stages. On the first day of training, mice were head-fixed briefly and given random water rewards to habituate them to the experimental environment. Behavioral experiments were conducted in a square behavioral chamber with a drinking spout directly in front of the mouth of the animal. Each mouse was kept in a covered elevated platform (custom-designed and 3D printed), with its head fixed by two stabilized clamps holding sidebars of the headpost. Heights of the tunnel and clamps were aligned before each session to ensure comfort. A steel tube was placed directly in front of the mouse. The spout and the metal sheet of the stage were connected to a contact lickometer that recorded the time and duration of licks at 2,000 samples/s (Slotnick, 2009). Head-fixed mice were allowed to voluntarily lick the spout (Guo et al., 2014). A 10% sucrose solution was delivered through the tube every 10 s controlled by MATLAB Psychtoolbox with a custom-made relay circuit.

After animals showed high levels of anticipatory licking, probe trials were inserted to assess the accuracy and precision with which mice timed target duration (Figure 3A). Sessions consisted of two probe trial types: rewarded and non-rewarded. The non-rewarded probe trials, also known as peak trials, allow measurement of internal timing of the behavior in the absence of actual reward feedback. During probe trials, the interval is three times longer than the target
duration (30 s), plus an additional random amount of time with a mean of 10 s with a Gaussian distribution. Peak trials represented roughly 20% of trials during a session (Figure 3B-C). To maintain performance, animals were provided with three rewarded trials in a row at which point the likelihood of a peak trial became 50%. This cycle was repeated after each peak trial. No external cue was provided to indicate the upcoming trial type. Mice were free to lick the spout at any time during the session.
Figure 3: Experimental setup and behavioral response during head-fixed timing task. (A) Head-fixed setup. Mice were placed on a raised platform and secured in place by head bars attached to chronic implants. A blunt-tipped needle is placed within licking distance, allowing for administration of a 10% sucrose reward using a solenoid under the control of an Arduino and MATLAB.
Psychtoolbox. (B) Schematic diagram of the reward schedules in the fixed-time-schedule task (left) and peak procedure (right), where 20% of rewards were omitted on probe trials. (C) Examples of behavioral data. Individual trial (top) and cumulative peri-stimulus time histogram (PSTH) (bottom) of licking behavior during rewarded (left) and peak (right) trials.

2.1.5 Local infusions

Mice received bilateral infusions of either 0.9% saline or muscimol (0.01 mg/ml) solution. All infusions contained a total volume of 400nL (i.e. 200nL per hemisphere) infused at a rate of 0.05 mL/hr. Infusion cannulas were left in place for 10 min post infusion. After removal of cannulas, animals were again given 10 min prior to starting any training. The condition order was counterbalanced across animals. Control sessions consisted of insertion of injector cannula, but without infusing any solution. The injectors were left in place for 10 min and animals were given 10 min after removal before starting any training.

2.1.6 Histology

After testing was completed, mice were deeply anesthetized with isoflurane and perfused with 0.1M PBS followed by 4% paraformaldehyde. Brains were post fixed in 4% paraformaldehyde for 24hrs followed by 30% sucrose. After sinking in the sucrose solution, brains were sliced coronally at 60 μm using a Leica CM1850 cryostat. For visualization of cannula placement, slices were stained with DAPI for 20 min at room temperature (Figure 4). Brightfield images were taken with an Axio Imager.M1 upright microscope.
(Zeiss). To confirm proper cannula placement, sections compared to images from a mouse brain atlas (Paxinos & Franklin, 2019).

**Figure 4: Infusion cannula localization.** Coronal slice with guide cannula tract and stereotaxic overlay (right). Infusion cannula tip (red asterisk) extended 0.5 mm from end of guide cannula. Localization of across all animals (left) with slice position measurements in the anterior-posterior axis relative to Bregma.

### 2.1.7 Data collection and statistical analysis

Experimental procedures were controlled by a MED Associates interface utilizing MED-PC software system. Lever presses were recorded in real time with 10 millisecond resolution. Session averaged data was analyzed using in-house Python scripts. All statistical analysis was performed in Prism 7.

For the session averaged data, lever presses for each trial were placed into 2s bins and collapsed across all trials. The cumulative pressing data was smoothed using a Savitzky-Golay filter and then fit using a Gaussian curve with the addition of a linear ramp accommodating positive skew, resulting from the temporal asymmetry of expected reward time relative to length of probe trials.
Fits were used to obtain peak times (accuracy) and peak spread (precision) as previously described (R. K. Cheng & Meck, 2007).

Lever pressing data for an individual trial was first placed into 1s bins. As the probe trials were of variable length with a minimum duration of 90s only the first 90 bins were included. The press-rate data was padded on both sides and smoothed using a median filter, followed by a Savitzky-Golay filter. The smoothed data was log normalized and then change points were detected using python ruptures library with a Pelt search method and “L2” cost function. To account for differences in press rate between animals, the penalty and median filter length values were allowed to vary.

In cases where session averaged data was unable to produce sufficient fits, the percentage of individual trials with temporal modulation was calculated. To assess whether lever pressing was temporally modulated on individual trials we compared nested maximum likelihood models of response data. This analysis is similar to that done when classifying 'time cells' using spike train data (Salz et al., 2016). First the maximum likelihood, assuming constant pressing during the duration of a probe trial, was calculated with a single constant term (a1):

\[ p_1(t; \theta_1) = a_1 \]

This model was then compared with a model that includes a temporally modulated term T, providing a two parameter Gaussian time field model with two parameters:

\[ p_2(t; \theta_2) = a_1 + a_2 T \]

where T is a Gaussian distribution parameterized by \( \mu \) and \( \sigma \):
\[
T(t; \sigma, \mu) = e^{-\frac{(t-\mu)^2}{2\sigma^2}}
\]

In order to be classified as a temporally controlled trial, model p2 must provide a better fit than model p1. The most parsimonious model was selected using a Bayesian information criterion (BIC). For a model to be preferred over the other \(\Delta\text{BIC} \geq 6\), a standard indicating strong evidence against the higher BIC model (Kass & Raftery, 1995).

2.2. Results

2.2.1 Inhibitions effects on 30s peak interval procedure

Before operant training began, mice \((n = 10, \text{C57BL/6})\) were implanted with guide cannulas and allowed two weeks to fully recover. After progressing to the final stage of training (see 2.1 Materials and Methods) animals were assigned an infusion schedule. The order in which a mouse received the three conditions was counterbalanced across subjects (table 1).

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C = control condition; S = 0.9% Saline condition; M = 0.01M muscimol condition

Session averaged response rates during non-rewarded probe trials were normalized in amplitude and fit using a Gaussian + Ramp function. Control
animals exhibited peak times at (26.33s ± 1.32s) for the 30s PI procedure, which was significantly lower than the 30s criteria, but in line with previous work in mice on the same task (Buonomano, Bramen, & Khodadadifar, 2009). Analysis of the peak functions revealed differences between muscimol and control conditions (Figure 5A). Moreover, the variance and peak time for session averaged data found that the muscimol condition demonstrated significant differences relative to control (t(18) = 9.35, p < 0.001; t(18) = 7.384, p < 0.001) and saline (t (18) = 7.004, p < 0.001; t(18) = 4.411, p = 0.016), while no significant differences were found between control and saline conditions (t(18) = 2.345, p = 0.248; t(18) = 2.973, p = 0.118) (Figure 5B).

Figure 5: Session averaged effects of muscimol. (A) Three traces of session averaged data for a single mouse under the control (black), 0.9% saline (grey), and muscimol (blue) conditions. Dashed line indicates peak time as defined by Gaussian + Ramp fit (see Session 2.1.6). (B) Comparison of peak time (left) and
To further elucidate the underlying factors contributing to the differences found in the session averaged data shown in Figure 1, we conducted a single trial analysis. For each trial, the start time, stop time, and duration of lever pressing was calculated (Figure 6A). This analysis revealed significant increases in the stop time ($t(81) = 6.217; p < 0.001; t(81) = 4.053, p < 0.001$) as well as duration of pressing ($t(81) = 5.431, p < 0.001; t(81) = 4.44, p < 0.001$) for the muscimol condition compared to control and saline conditions, respectively (Figure 6B).

**Figure 6: Effects of muscimol on individual trials.** (A) Session raster plots of probe trials under saline (top) and muscimol (bottom) conditions. Each black tick
represents a single lever press with calculated start (green) and stop (red) times of the 'high state' for each trial. (B) Comparison of start, stop, and duration of individual trial data across infusion conditions. *** p < 0.001

The distribution of inter-press-intervals (IPIs), a metric of pressing structure, shows qualitatively similar profiles between muscimol (0.69 ± 0.0044 s, SD = 0.398s) and saline (0.65 ± 0.0033, SD = 0.332s) conditions (Figure 7A) making changes in motor output an unlikely confound. Additionally, no significant difference in total lever presses was found between groups (Figure 7B), indicating increases in response variance could not be solely explained by differences in excessive lever pressing in muscimol animals. However, the proportion of presses during trials (i.e. auditory cue “on”) compared to total presses was significantly lower for the muscimol condition compared to both control (t(18) = 7.871, p < 0.001) and saline (t(18) = 6.762, p < 0.001). This measurement, known as the elevation ratio, indicates the level of discrimination an animal has between the cue and ITI (Figure 7C). Therefore, while the muscimol doses given in this study did not significantly change overall lever pressing, it did alter the animal’s ability to distinguish between which discrete phases during the task were associated with rewarded presses.
Figure 7: Muscimol does not modify pressing behavior. (A) Density plot of the inter-press-interval between presses during the ‘high-state’ across all subjects for the saline (grey) and muscimol (blue) conditions demonstrating qualitatively similar profiles. (B) Total number of lever presses produced at any time during a session across control (white), saline (grey), and muscimol (blue) conditions. (C) The elevation ratio across all conditions defined as the percent of presses occurring during a trial relative to the total number of presses produced at any time within a session. *** p < 0.001
2.2.2 Inhibitions effects on 10s head-fixed timing task

To assess the generality of the results demonstrated in the 30s PI procedure, animals were trained on an additional 10s head-fixed timing task. Of the original 10 subjects, 2 were excluded as they were unable to obtain task competency. Within the task, motor output was quantified in the form of licking activity at three time points for all peak trials. The first being the phase prior to reward delivery referred to as anticipatory licking, the second was the time period following the reward delivery denoted as consummatory licking, and lastly was licking activity over the peak trial itself. Contrary to the operant results, repeated measures ANOVA found significant differences in motor output during the anticipatory (F(2,14) = 11.18, p < 0.01) and during the peak trial (F(2, 14) = 4.644, p < 0.05). A post-hoc Tukey test revealed significantly lower rates of anticipatory licking during muscimol condition than control and saline conditions and significantly lower total licking over the peak trial for the muscimol condition than control with the saline condition in between the two (p < 0.05). However, there was no significant effect by condition in lick rates during the consummatory phase (F(2, 14) = 1.286, p = 0.3072), indicating that muscimol’s effects on motor output are not due to general slowing of motor output per se, but seemingly specific to actions dependent on internal timing (Figure 8A). Session averaged data captures these qualitative differences in self-initiated licking compared to licking in the presence of a 10% sucrose solution reward (Figure 8B).
Figure 8: Muscimol effects on activity during head-fixed timing task. (A) The effects of experimental condition on licking activity during the anticipatory (left), consummatory (middle), and peak trial (right). (B) Three traces of session averaged data for a single mouse under the control (black), saline (grey), and muscimol (blue) conditions. * p < 0.05, ** p < 0.01
Despite the striking difference in session performance, raster plots of individual trials show that animals are in fact licking during the muscimol condition (Figure 9A), as also evidenced by the lack of significant differences between saline and muscimol peak trial licking. The exceedingly flat licking profile for the muscimol condition made traditional means of quantification impossible, with only 2 of 8 subjects producing detectable peaks. Therefore, a novel analytical method for assessing timing behavior was adopted. In order to quantify the temporal specificity of lever presses on individual trials nested maximum likelihood models were applied as previously done for assessing time cell spike trains (Salz et al., 2016). The muscimol condition saw a dramatic 22% decrease (42% to 20%) in temporally controlled trials. This was accompanied by a 27% increase (28% to 55%) in non-temporally controlled trials (Figure 9B). The number of unclassified trials remained relatively similar with only a 5% decrease (30% to 25%). To see how these findings compared with performance on the 30s PI procedure, the same analysis was conducted on the operant data and found the muscimol condition saw a 15% decrease (78% to 63%) in trials that contained temporally modulated pressing. This was accompanied by an 11% increase (14% to 25%) increase in non-temporally modulated trials and 4% increase (8% to 12%) in unclassified trials (Figure 9C).
Figure 9: Individual trial analysis of head-fixed timing performance. (A)

Session data for a single animal during saline (top) and muscimol (bottom) conditions. Dashed red lines indicate where the last reward was presented (t = 0) and where the animal expected the omitted reward to be (t = 10s). Each lick is
represented by a black dash. (B) Delta BIC values, indicating the degree of
temporal control, for licking activity for each trial across all animals for saline (top)
and muscimol (bottom) trials during head-fixed timing task. (C) Same as in (B)
except for trials of the 30s PI procedure

2.3 Discussion

Using reversible pharmacological inhibition of the MD we found deficits in
both temporal precision and accuracy in session averaged data. Analysis of
individual trials demonstrated these effects to be the result of significantly later
stop times for the pressing high-state and significantly longer pressing bouts.
Training on a head-fixed timing task established the effects of MD inhibition were
not limited to operant timing procedures and is likely a part of a generalized
timing network. Additionally, in the head-fixed task, which is more reliant on
internally driven timing mechanisms, the effects of MD inhibition were more
robust. These findings provide some of the first evidence showing involvement of
the mediodorsal thalamus in supporting proper timing behavior over multiple
seconds.

A sizable collection of research in the field of time and time perception has
been dedicated to the involvement of the frontal cortex in interval timing. Notably,
recent pharmacological work found comparable results to the ones described
here through inactivation of the prelimbic (PL) cortex in the rat. Rats in this study
demonstrated decreased precision, significant increases in the stop time of
individual trials, and decreased elevation ratios (Buhusi et al., 2018). The
importance of these results is twofold. First, they are in line with many of the findings from the current study, which also found significant differences in the three aforementioned measurements. Second, the prelimbic cortex is one of the primary regions of the frontal cortex reciprocally connected to the MD. Together, this provides incredible insight into the mechanism through which the MD influences timing behavior. Recent work dissecting the interactions between MD afferents to the mPFC found neurons in the mPFC have sequential firing patterns during the delay phase of a working memory task similar to the sequential activation of ‘time cells’ in the mPFC during timing tasks (Tiganj et al., 2017). Importantly, terminal inactivation of MD projections eliminated the delay-dependent activation in the mPFC and led to deficits in performance (Bolkan et al., 2017).

It is important to note that, while the current work does capture many of the key findings from inactivation of the PL cortex, it does not simply reproduce those results. Unlike with inactivation of the PL cortex, the current study did not find a significant decrease in the start time of individual trial high-states. In addition, the current study found a significant rightward shift in peak time. Differences in analytical methods could potentially account for the discrepancy between the studies, in relation to changes in accuracy, as the greatest difference on individual trials was the change in stop times. Therefore, the rightward shift found in the current study is potentially a manifestation of the asymmetry related to the criterion time within the duration of a probe trial (i.e., only ½ of the way into the trial). Conversely, the MD influences cortical activity in
additional regions of the frontal cortex such as the anterior cingulate cortex (ACC) (Delevich, Tucciarone, Huang, & Li, 2015) an area known for encoding signal duration information (Matell et al., 2003). The disruption of information transferred and modulated via the indirect cortico-thalamo-cortical pathway, opposed to the direct cortico-cortical pathway (Jones, Groenewegen, & Witter, 2005), may also contribute to the differences between studies.

The current work is not the first to demonstrate striking behavioral overlap with lesions of frontal cortical regions, particularly the PL. Excitotoxic lesions of the MD, as well as the PL have previously demonstrated disruptions of spatial working memory, but only if the rules required for successful execution of the task involves deviations from known strategies (Shaw & Aggleton, 1993). Therefore, further validating the strong relationship between proper functioning in these two brain regions and proper behavioral output across multiple aspects of cognition.

A surprising discovery was the difference in sensitivity to MD inhibition between the two tasks used in the current study. While a variant of the PI procedure, the head-fixed timing task does have fundamental differences related to reward type, behavioral response, and trial construction all of which may potentially contribute to the differences observed. Of these differences, the most probable contributor is the absence of any external stimuli outside of the reward. Indeed, non-temporal components related to task parameterizations can influence timing behavior. The modality of the timing signal can impact the perceived duration of a stimulus with auditory cues being judged, on average, as
lasting longer than visual (Droit-Volet, Meck, & Penney, 2007; Lustig & Meck, 2011; Shi, Church, & Meck, 2013). Moreover, the sensitivity to a particular stimulus modality is capable of being individually modulated (Cheng, Scott, Penney, Williams, & Meck, 2008). Stimulus intensity has also been shown to shape timing behavior. Be it size, loudness, or luminance (W. J. Matthews, Stewart, & Wearden, 2011), the greater the stimulus intensity the longer the duration is perceived. The differences between stimulus properties and timing have been theorized to be associated with the different levels of attentional resources allocated to the stimulus (Penney, 2003). The more salient the cue, the more of an animal's attention the cue is able to garner, and the more focused an animal can be on the task. The lack of external cues on the head-fixed task makes it easier for animals to lose focus and disengage as they rely solely on the temporal profile of the task as the only information conveying the time of a subsequent reward is the elapsed time since the previous one. Additionally, the MD plays an important role in sustained attentional control (Schmitt et al., 2017), therefore, it's inactivation further comprises timing performance. This likely puts far greater strain on internal timing mechanisms as the animal is unable to rely on sensory input like that found in the traditional discrete trial format of the 30s PI procedure.

This work is also the first to the authors knowledge that adapted the nested maximum likelihood models for ‘time cell’ classification for behavioral analysis. This form of single-trial analysis is particularly valuable, compared to previous used analytical methods, as it provides a quantitative assessment of
temporal structure on a given trial. While this level of inquiry is not always necessary, in manipulation studies that can potentially produce major deficits in timing behavior, being able to quantify the effects can be highly valuable over qualitative appraisal. Additionally, it provided a tool for comparing the difficulty of two tasks. In the control conditions, we found a far greater number of trials that could be classified as temporally controlled (i.e. Gaussian) on the 30s PI procedure than we did in the 10s HF task indicating temporal control in the prior task. As the threshold for identification of either Gaussian or Uniform is somewhat flexible, the exact percent of trials in each category is less important than the difference between conditions. Though previous analytical methods have been used for comparing performance across tasks, they have relied on the quantification of the session averaged data.

However, there is reason to believe that session data may not be the best indication of difficulty. A common measurement of task difficulty, the coefficient of variation (CV), is the ratio of peak spread to peak time (Lewis & Miall, 2009). With this method, if the peak spread increases for a particular task and the peak time remains the same, the CV value increases, and the task is presumed to be more difficult. Based on this measurement both the PI procedure and head-fixed task would be judged as equally difficult as they both have CV values of roughly 0.60 (Buhusi et al., 2009; Toda et al., 2017). This equality of difficulty is incongruent with the fact that mice have difficulty acquiring competency on the head-fixed task for durations over 20s, though routinely learn durations of 30s or greater on the PI procedure. Moreover, a skeletonized version of the PI
procedure which uses empty intervals and is more analogous to the head-fixed task in that regard takes many more days of training for mice to acquire. Together the comparison of temporally modulated trials using nested maximum likelihood models may provide a better evaluation of task difficulty.

Despite the physical localization of the pharmacological modulation, the temporal dynamics of the muscimol are quite broad lasting the entirety of the session. Therefore, this technique is incapable of elucidating how the MD may differentially contribute to different phases of an individual trial. Specifically, as previously discussed (see Section 1.1.2), individual trials consist of a low-high-low activity profile and how the MD may influence the time at which an animal transitions from one state to the next remains unanswered. In order to address these limitations implementation of techniques with greater temporal control such as optogenetics would be required.
3. Optogenetic inhibition of mediodorsal thalamus and interval timing behavior

In order to understand how inhibition of the MD thalamus may contribute to interval timing behavior on a finer temporal scale. Optogenetics provides an indispensable technique for cell-specific, temporally precise modulation of neural activity (Boyden, Zhang, Bamberg, Nagel, & Deisseroth, 2005). The temporal precision, in the milliseconds range, afforded by optogenetic techniques makes them well suited for understanding timing behavior in a way pharmacological manipulation cannot.

Optogenetics involves inducing the expression of a microbial opsin, a natural light-sensitive ion-transporting membrane protein, in neurons allowing for their activity to be controlled by light. The expression pattern, modulation, and visualization are dependent on four key factors: viral vector type, viral promoter, opsin gene, and fluorescent tag. The most common viral vector for introducing opsins into mouse neural tissue is adeno-associated viral vectors (AAV) (Monahan & Samulski, 2000). The effectiveness of AAV as an opsin delivery vector has spurred the development of various stereotypes all with different patterns of transduction (Burger et al., 2004; Taymans et al., 2007). The promoter further influences the targeting specificity, with cell-type-specific promoter allowing for expression to be restricted to a particular neural sub-population. Additionally this can be done through the use of transgenic animals with cell-type-specific expression of a recombinase such as Cre and a double floxed inverted opsin (Sohal, Zhang, Yizhar, & Deisseroth, 2009). The opsin gene
dictates the effect light has on the electrical activity of the transduced cell. They can be broadly grouped into excitatory or inhibitory based on their mechanism of action, though additional kinetic and spectral attributes are important to consider (Yizhar, Fenno, Davidson, Mogri, & Deisseroth, 2011).

For the experiments on chapter 3, an AAV5-EF1α-DIO-eNpHR3.0-eYFP construct was chosen with the use of transgenic mice expressing Cre-recombinase behind the vesicular glutamate transporter promoter (Vglut2-IRES-Cre) present in excitatory neurons in the rodent MD (Hur & Zaborszky, 2005; Yuge et al., 2011). Halorhodopsin (eNpHR3.0) is a light-activated chloride pump that hyperpolarizes cells with application of yellow light (589-nm) (Gradinaru, Thompson, & Deisseroth, 2008; Lanyi & Oesterhelt, 1982). This allows for selective inhibition of excitatory neurons in the MD.

3.1 Materials and Methods

3.1.1 Viral Constructs

AAV5-EF1α-DIO-eNpHR3.0-eYFP and AAV5-EF1α-DIO-eYFP were obtained from the Duke Vector Core.

3.1.2 Animals

Data was collected from adult Vglut2-IRES-Cre mice (n = 11). In all behavioral experiments, mice were maintained on a 12:12 light cycle. Housing and food restriction procedures are identical to those in the previous study and outlined in section 2.1.1. All experimental procedures were approved by the Duke University Institutional Animal Care and Use Committee.
3.1.3 Surgery

Mice were anesthetized with 1.0 to 2.0% isoflurane mixed with 1.0 L/min of oxygen for surgical procedures and placed into a stereotactic frame (David Kopf Instruments, Tujunga, CA). Adult Vglut2-IRES-Cre mice were randomly assigned to Vglut2::eNpHR3.0 (n = 6) or Vglut2::eYFP groups (n = 5). Virus was bilaterally microinjected into the MD (0.3 μL each hemisphere, AP: −1.35 mm, ML: ± 1.48 mm, DV: −3.3 mm from skull surface, at 20° from vertical). All measurements are relative to bregma. Mice were bilaterally implanted with custom made fiber optics aimed directly above the MD (AP: −1.35 mm relative to bregma, ML: ± 1.48 mm, DV: −3.1 mm from skull surface, at 20° from vertical). Fibers were secured in place with dental acrylic adhered to skull screws. Mice were singly housed during recovery for at least two weeks before training began.

3.1.4 Peak Interval Procedure

Training for the 30s PI procedure was performed as previously outlined (see Section 2.1.3), with the addition of laser-on (ON) and laser-off (OFF) trial. Each trial was randomly selected to either ON or OFF at trial onset, and both FI and non-rewarded probe trials were eligible.

3.1.5 Optogenetic Inhibition

Optical inhibition began after an animal had progressed to the PI procedure and developed a distinctive peak in pressing activity centered around 30 s. Mice were connected to a 589-nm DPSS laser (Shanghai Laser) via fiber optic cables (Doric: core = 200m; NA = 0.22) and placed inside the testing
chamber. A rotating optical commutator (Doric) divided the beam (50:50) permitting bilateral inhibition. Inhibition (6-8 mW; constant; 15s, 10s, or 5s duration) was delivered at three time points: cue onset (t = 0), 15s after cue onset (t = 15), or 60s after cue onset (t = 60). The first two conditions occurred on both FI and probe trials, while the final condition only occurred on probe trials. The time of stimulation was held constant within a session but varied across sessions.

3.1.6 Histology

After testing was completed, mice were deeply anesthetized with isoflurane and perfused with 0.1M PBS followed by 4% paraformaldehyde. Brains were post fixed in 4% paraformaldehyde for 24hrs followed by 30% sucrose. After sinking in the sucrose solution, brains were sliced coronally at 60 μm using a Leica CM1850 cryostat. For visualization of viral expression and fiber placement, fluorescent images were taken (Figure 10A-B) with a Z10 inverted microscope (Zeiss). To confirm proper fiber placement, sections compared to images from a mouse brain atlas (Paxinos & Franklin, 2019).
3.1.7 Data Collection and Analysis

Data collection and analysis was performed as previously outlined (see Section 2.1.7), with a few notable differences. Due to half of the probe trials being in either ON or OFF condition, two sessions for each time point were concatenated together in order to obtain a sufficient number of trials.

3.2 Results

While pharmacological inhibition of the MD demonstrated its importance to proper timing behavior, how the MD activity contributes to individual behavioral phases during a PI procedure remains unanswered. A given trial can be divided into three phases: initial “low-state” followed by a pressing “high-state” and concluding with a second “low-state” (R. M. Church et al., 1994). To investigate the contributions of MD to behavior with higher temporal resolution, we used

Figure 10: Expression of eNpHR3.0-eYFP in MD. (A) Schematic of surgical procedure. (B) Expression of eNpHR3.0-eYFP in a Vglut2-IRES-Cre mouse.
optogenetics to inactivate the MD. We used a Cre-dependent halorhodopsin (DIO-eNpHR3.0) in vGlut2-Cre mice to selectively inhibit vGlut2+ projection neurons in the MD (Zhang et al., 2007). This manipulation provides more spatially and temporally selective inactivation of the MD. Spatially, it is limited to the glutamatergic projection neurons; temporally it can be used at specific time points during the trial. We selected three time points for inhibition (0s, 15s, and 60s). Each time point occurs primarily during one of the three phases. Inhibition was constant over 15s as this has previously been shown to effectively disrupt prefrontal dependent processes such as working memory maintenance (Bolkan et al., 2017).

Performance on non-laser trials was consistent with previous work. Peak time (25.53s ± 0.719s) was significantly lower than 30s, but not significantly different than control data from muscimol experiments (t(14) = 0.4416, p = 0.6655), nor was the peak variance (t(14) = 0.841, p = 0.4145). Therefore, despite having MD inhibition on 50% of the reward trials, there is likely no residual influence on non-laser peak trials. When optical inhibition was concurrent with the onset of the trial, animals produced a systematic delay in the onset of lever pressing, evidenced by a rightward shift in peak time. However, there was no significant difference in temporal variance (Figure 11A-B). Single trial analysis demonstrated the rightward shift to be a product of delays in the start (t(10) = 4.419, p = 0.003) and stop (t(10) = 2.392, p = 0.040) of the pressing high-state with no changes in the duration of pressing (t(10) = 0.6533, p = 0.370: Figure 11C). A 2-dimensional density plot shows qualitative similarities
in the distribution of start and stop times (Figure 11D). Despite having significantly different means, an F-test for equality of variance found no significant differences between the two conditions for start times (F(140, 124) = 1.06, p = 0.5669) or stop times (F(124, 140) = 1.303, p = 0.1284).

Figure 11: Optogenetic inhibition of MD at trial onset. (A) Individual trial raster (top) and averaged data (bottom) across two sessions with optical inhibition at t = 0s. (B) Peak time (left) and variance (right) of fits to session
averaged data across stimulation conditions. Both individual (blue) and collapsed data (grey) are presented. (C) Individual trial data showing start, stop, and duration of individual trials across stimulation (blue) and non-stimulation (grey) trials. (D) 2-dimensional density plot of start and stop times. ** p < 0.01, *** p < 0.001

To assess how MD inhibition modulates behavior during the pressing high-state, inhibition was started 15s into the trial, when mice were likely to have already started to engage in pressing. As with optical inhibition at onset, animals produce rightward shifts in peak pressing, accompanied by an increase in peak variance (Figure 12A-B). Single trial analysis was again done in order to evaluate the contributing factors to differences found in session averaged data between conditions (Figure 12C). Contrary to the effects seen with inhibition at trial onset, inhibition at t = 15s produced significant delays in pressing stop times ($t(10) = 3.599, p = 0.005$) as well as increases in the duration of the pressing high-state ($t(10) = 2.889, p = 0.008$) with no significant differences in start times ($t(10) = 0.2602, p = 0.280$).
Figure 12: Optogenetic inhibition of MD during trial 'high-state'. (A) Individual trial raster (top) and averaged data (bottom) across two sessions with optical inhibition at t = 15s. (B) Peak time (left) and variance (right) of fits to session averaged data across stimulation conditions. Both individual (blue) and collapsed data (grey) are presented. (C) Individual trial data showing start, stop, and duration of individual trials across stimulation (blue) and non-stimulation (grey) trials. ** p < 0.01

In addition to the increases in mean length of pressing, how inhibition of the MD affected the variability of press duration was investigated through assessment of Gaussian fits of press duration histograms (Figure 13A). An extra sum-of-squares F-test found the variance of the inhibition condition’s best fits was significantly greater than that of non-inhibition trials (F(1, 32) = 15.14, p = 0.0005). As the start of the lever pressing bout (high state) can vary from trial to
trial, a linear regression was performed between the bout duration and total amount of inhibition as a fraction of the possible 15s (Figure 13B). When only part of the inhibition occurred during pressing, no distinction was made between whether inhibition occurred at the beginning or end of the pressing. The fraction of inhibition showed a strong positive correlation with duration of the lever pressing bout ($R^2 = 0.6136$, $p < 0.001$).
Figure 13: Optogenetic inhibition of MD effects on 'high-state' duration. (A) Histogram and Gaussian fits of the duration of individual trial pressing activity for stimulation (blue) and non-stimulation (grey) trials demonstrating increased variance and average time for stimulation trials. (B) Linear-regression relating the duration of a 'high-state' press bout to the length of MD inhibition that occurred during the bout.
The final time point for optical inhibition, $t = 60s$, occurs after the animal has finished pressing. As laser stimulation occurs after the expected start and stop time of individual trials, inhibition at this point has no significant effects on timing performance as indicated by either session averaged (Figure 14A-B) or single trial (Figures 14C) analysis.

**Figure 14: Optogenetic inhibition of MD after 'high-state'.** (A) Individual trial raster (top) and averaged data (bottom) across two sessions with optical inhibition at $t = 60s$. (B) Peak time (left) and variance (right) of fits to session averaged data across stimulation conditions. Both individual (blue) and collapsed data (grey) are presented. (C) Individual trial data showing start, stop, and
duration of individual trials across stimulation (blue) and non-stimulation (grey) trials.

However, inhibition of the MD at t = 60s led mice to re-engage with the task on a number of trials, often referred to as ‘rebound’ pressing (Figure 15A). A paired t-test found mice exhibited a significant increase in rebound pressing during inhibition trials (t(5) = 3.238, p = 0.0181). This effect was not found in eYFP-control mice (t(4) = 0.0308, p = 0.488). To see if there was a change in the number of rebound trials over the course of a session, each session was divided into fifths and the percent of rebound trials was calculated (Figure 15B). A repeated-measures ANOVA found no significant difference in the percent of trials with rebound pressing across bins (F(11, 44) = 1.973, p = 0.0551).

![Graphs showing rebound pressing after MD inhibition at t = 60s.](image)

**Figure 15: Rebound pressing after MD inhibition at t = 60s.** (A) Percent of trials with rebound activity within a session eYFP controls and eNpHR3.0 mice. (B) Change in rebound pressing across individual sessions. * p < 0.05
The length of optical inhibition was systematically modulated to see how the length of inhibition affected the results seen with 15s of inhibition. For inhibition at trial onset, the magnitude of the rightward shift held a linear relationship to the duration of optical inhibition, with 15s of inhibition leading to the greatest shifts and 5s producing no deviation in peak time (Figure 16A). Significant linear relationships were also observed in primary findings from the other inhibition time points. For inhibition at $t = 15s$, a one-way repeated measure ANOVA shows a significant treatment effect ($F(1.31, 5.238) = 30.21, p = 0.002$) with longer durations of MD inhibition leading to increases in variance. A Holm-Sidak multiple comparison post-hoc test found 15s inhibition produced significantly greater increases in variance than 5s or 10s of inhibition (Figure 16B). These results comport with the single-trial results showing the increase on individual trials scales with the length of inhibition during the pressing phase. Lastly, inhibition at time point $t = 60s$ demonstrated a significant treatment effect in the percent of trials that exhibited rebound pressing (Figure 16C). A two-way repeated measures ANOVA revealed a significant main effect of stim length on percent of rebound trials ($F(2, 8) = 5.199, p = 0.036$). Additionally, a Bonferroni corrected multiple comparison post-test found inhibition lengths of 15s ($t(8) = 5.526, p = 0.002$) and 10s ($t(8) = 4.435, p = 0.007$) result in a significantly greater percent of rebound trials than non-inhibition trials. Two lines of evidence suggest this is unlikely to be explained by confounding factors such as visually perceiving light from the laser. First, eYFP-control mice did not exhibit any changes in rebound pressing (Figure 14A) and second, the 5s condition did not produce
significantly more rebounding than non-laser trials, despite the same length of a visual distractor having been previously shown to modify timing behavior in rats performing a 30s PI procedure (Buhusi & Meck, 2006).

**Figure 16: Relationship between inhibition duration and effect magnitude.**

(A) The percent change in session average peak time relative to non-stim trials when inhibition starts at t = 0s. (B) The percent change in session average variance. (C) The percent of trials with rebound.
variance relative to non-stim trials when inhibition starts at t = 15s. (C) The percent of trials in which rebound pressing was detected when inhibition was started at t = 0s. * p < 0.05, ** p < 0.01, *** p < 0.001, ## p < 0.01

To address potential confounds related to light delivery, AAV5-EF1α-DIO-eYFP controls (n = 5) were tested on the 30s PI procedure using identical optogenetic conditions. Control mice did not exhibit any significant differences across trial types (Figure 17A-C).
Figure 17: Behavior of eYFP control mice. (A) Individual trial raster (top) and averaged data (bottom) across two sessions with optical inhibition at time points: 0s, 15s, and 60s. (B) Peak time (left) and variance (right) of fits to session averaged data across stimulation conditions. Both individual (blue) and collapsed data (grey) are presented. (C) Individual trial data showing start, stop, and duration of individual trials across stimulation (blue) and non-stimulation (grey) trials.
3.3 Discussion

Using optogenetic techniques to inhibit the MD at distinct time points within individual trials, we demonstrated dissociable effects on timing behavior based on the time of inhibition. Inhibition at the onset of a trial (t = 0s) delayed the start time and stop time of lever pressing leading to rightward shifts in the peak time when activity was averaged over multiple sessions. When inhibition was initiated 15s into the trial the duration in which the animal engaged with the level increased. Over the session this caused effects in precision and accuracy, leading to increases in peak spread and a rightward shift in peak time, respectively. Lastly, inhibition after the pressing ‘high-state’ (t = 60s) increased the probability that an animal would re-engage with the lever. This work represents the first time the effect of MD inhibition on discrete phases of individual trials during an interval timing task has been observed, providing important insight into neural underpinnings of time perception.

While the behavioral effects of MD inhibition at t = 0s and t = 15s are distinct, they point to a similar underlying mechanism. Both can be seen as perseverating in the current state and delaying the transition into the next state. These types of delays can occur either when internal timing mechanisms are slowed (i.e. clock speed), when deficits in attentional mechanisms temper the rate temporal information is integrated, or when the memory of the duration is altered (Buhusi & Meck, 2005). There are two main reasons to suppose the results found here are a result from changes in attentional gating. the frontal cortex, which is intimately connected to the MD, has been shown to be important
for attention allocation during timing tasks (Lake & Meck, 2013; Olton, Wenk, Church, & Meck, 1988). Conversely, clock speed and temporal memory are tied to the basal ganglia and hippocampus, respectively. While these areas do have connections to the MD they are not as robust as those with the frontal cortex. Behaviorally, the consequences of disruptions to these components of timing are best described by attentional mechanisms as well. Challenges to temporal memory result in latent changes and are not representative of within trial modification (Meck, 1996). Additionally, clock speed effects cannot explain the rebound pressing found with inhibition at t = 60s.

The findings that the length of stim during pressing correlates with the length of pressing supports a model of timing in which each stage of the task is timed independent of the other. That is, the animal will time when it should begin pressing and then when to stop, sequentially. This serial or at least quasi-serial interpretation of timing has been outlined in theoretical models of interval timing such as the behavioral theory of timing (BeT) (Killeen & Fetterman, 1988, 1993), where animals learn behavioral sequences that act as proxies for properly timing. However, this is in conflict with early analysis of individual trial studies that found support for parallel processing theories, most notably Scalar Expectancy Theory (SET) (John Gibbon, 1977; J. Gibbon & Church, 1990). Previous work had found the covariance pattern between start and stop times during the ‘high-state’ supported the use of a single temporal sample from memory with different decision thresholds for when to start and stop pressing (R. M. Church et al., 1994; J. Gibbon & Church, 1992). However, the ability to maintain the necessary
covariance pattern, among other timing behavior phenomenon, does not necessitate a parallel processing, as theories based on memory decay have demonstrated (Staddon & Higa, 1999).

In order to analyze the percent of trials with rebounding activity a novel algorithm was implemented, as previous methods for detecting such behavior suffered from two main weaknesses. First, they relied on brute force search methods (Matell et al., 2006) that are computationally expensive and slow. More important to the current work, methods for detecting multiple 'high-states' have required a presumption of multiple 'high-states' existing, often based on the particular task. For example, during a PI procedure with a gap in the trial signal, animals will start pressing at signal onset, pause during the signal gap, and reinstate pressing when the signal returns. Therefore, it is known that all trials will have two 'high-stats' (Swearingen & Buhusi, 2010). However, for the purposes of detecting rebound activity it is important that the algorithm does not require such an assumption. The algorithm developed here provides a fast, effective method for detecting even complex activity profiles with minimum parameterization.

Despite the notable findings presented here, there are many outstanding questions. Though the behavioral effects are best described by challenges to attentional processes, further work is required to validate this theory. One important study is the parametric modulation of the trial signal. Previous work has shown the desirability of the trial signal relative to the inter-trial-interval has a significant effect on temporal integration (Buhusi, Sasaki, & Meck, 2002). Additionally, the MD receives extensive inputs from cortical and subcortical brain
regions, as well as projects broadly to the frontal cortex. Dissecting how various regions associated with the MD contribute to the current findings is vital. In all, the work here is a significant step forward in understanding the role of the MD in time perception and its likely role in supporting attentional processes during interval timing.
4. Optical Inhibition of localized mediodorsal thalamic projections to prelimbic cortex

The MD has broad projections throughout the frontal cortex. Therefore, whether the effects of MD inhibition on time perception are mediated through a single region connected to the MD or related to its role as a relay and integrator between regions is unknown. Since its inception, advancements in optogenetic techniques allow for retrograde targeting (Li, Ryu, Krueger, Heldt, & Albritton, 2012) and evaluation of these potential explanations.

Extensive research has been dedicated to understanding the involvement of the PL cortex in interval timing behavior (Buhusi et al., 2018; J. Kim et al., 2009; A. R. Matthews, He, Buhusi, & Buhusi, 2012). As substantial reciprocal connections exist between the MD and PL cortex, investigation into the interactions between these regions posed a potentially fruitful avenue. Furthermore, many of the previous findings from previous chapters agree with pharmacological modulation of the PL cortex.

4.1 Materials and Methods

4.1.1 Viral Constructs

AAV5-EF1α-DIO-eNpHR3.0-eYFP and AAV5-Retro2- EF1α-CRE were obtained from the Duke Vector Core.

4.1.2 Animals

Data was collected from adult wild-type mice (C57BL/6; n = 5). In all behavioral experiments, mice were maintained on a 12:12 light cycle. Housing
and food restriction procedures are identical to those in the previous study and outlined in section 2.1.1. All experimental procedures were approved by the Duke University Institutional Animal Care and Use Committee.

### 4.1.3 Surgery

Mice were anesthetized with 1.0 to 2.0% isoflurane mixed with 1.0 L/min of oxygen for surgical procedures and placed into a stereotactic frame (David Kopf Instruments, Tujunga, CA). AAV5-Retro2-\(\text{EF1}\alpha\)-CRE was bilaterally microinjected into the PL (0.3 μL each hemisphere, AP: +2.2 mm, ML: ± 0.2 mm, DV: −2.0 mm from skull surface). Subsequent injections of AAV5-\(\text{EF1}\alpha\)-DIO-eNpHR3.0-eYFP was bilaterally microinjected into the MD (0.3 μL each hemisphere, AP: −1.35 mm, ML: ± 1.48 mm, DV: −3.3 mm from skull surface, at 20° from vertical). Custom made fibers were also implanted bilaterally aimed directly above the MD (AP: −1.35 mm relative to bregma, ML: ± 1.48 mm, DV: −3.1 mm from skull surface, at 20° from vertical). Fibers were secured in place with dental acrylic adhered to skull screws. Mice were singly housed during recovery for at least two weeks before training began. All measurements are relative to bregma.

### 4.1.4 Peak Interval Procedure

Training for the 30s PI procedure was performed as previously outlined (see Section 2.1.3), with the addition of laser-on (ON) and laser-off (OFF) trial. Each trial was randomly selected to either ON or OFF at trial onset, and both FI and non-rewarded probe trials were eligible.
4.1.5 Optogenetic Inhibition

Optical inhibition began after an animal had progressed to the PI procedure and developed a distinctive peak in pressing activity centered around 30 s. Mice were connected to a 589-nm DPSS laser (Shanghai Laser) via fiber optic cables (Doric: core = 200m; NA = 0.22) and placed inside the testing chamber. A rotating optical commutator (Doric) divided the beam (50:50) permitting bilateral inhibition. Inhibition (6-8 mW; constant; 15s, 10s, or 5s duration) was delivered at three time points: cue onset (t = 0), 15s after cue onset (t = 15), or 60s after cue onset (t = 60). The first two conditions occurred on both FI and probe trials, while the final condition only occurred on probe trials. The time of stimulation was held constant within a session but varied across sessions.

4.1.6 Histology

After testing was completed, mice were deeply anesthetized with isoflurane and perfused with 0.1M PBS followed by 4% paraformaldehyde. Brains were post fixed in 4% paraformaldehyde for 24hrs followed by 30% sucrose. After sinking in the sucrose solution, brains were sliced coronally at 60 μm using a Leica CM1850 cryostat. For visualization of viral expression and fiber placement, fluorescent images were taken (Figure 18A-B) with a Z10 inverted microscope (Zeiss). To confirm proper fiber placement, sections compared to images from a mouse brain atlas (Paxinos & Franklin, 2019).
Figure 18: Expression of eNpHR3.0-eYFP in MD. (A) Schematic of surgical procedure. (B) Expression of eNpHR3.0-eYFP in the MD using retrograde targeting in a C57BL/6 mouse.

4.1.7 Data Collection and Analysis

Data collection and analysis was performed as previously outlined (see Section 2.1.7), with a few notable differences. Due to half of the probe trials being in either ON or OFF condition, two sessions for each time point were concatenated together in order to obtain a sufficient number of trials.

4.2 Results

After recovery from surgery, wild-type mice (C57BL/6; n = 5) were tested on the 30s PI procedure as outlined in the previous chapter. However, unlike inactivation of all MD cell bodies, optical inhibition of only PL projecting cells did not reproduce the changes in timing behavior. While laser stimulation was
performed at time points t = 0s, 15s, and 60s, only the t = 0s condition resulted in significant findings. Paired t-tests on the parameters of fits to session averaged data demonstrated a significant rightward shift in peak (t(4) = 3.742, p = 0.0201) but no changes in peak variance (t(4) = 0.3994, p = 0.71; Figure 19A-B). Interestingly, this finding was not accompanied by changes in individual trial performance (Figure 19C). This is the first example of changes in session averaged performance conflicting with the underlying constituent trials.

Figure 19: Inhibition of MD projections to PL at trial onset. (A) Individual trial raster (top) and averaged data (bottom) across two sessions with optical inhibition at t = 0s. (B) Peak time (left) and variance (right) of fits to session averaged data across stimulation conditions. Both individual (blue) and collapsed
data (grey) are presented. (C) Individual trial data showing start, stop, and duration of individual trials across stimulation (blue) and non-stimulation (grey) trials. *p < 0.05

While the rightward in session averaged performance was significant, it was not of comparable magnitude to the shifts found with 15s inhibition of all MD cells ($t(4) = 3.707, p = 0.006$). Therefore, while inhibition of a subpopulation of MD cells has the same qualitative effects as total inhibition, it is quantitatively mitigated. Comparisons of the shifts in peak time from inhibition at $t = 0s$ and variance from inhibition at $t = 15s$ found them to be qualitatively similar in magnitude (Figure 20 A-B). Surprisingly, not all the conditions showed these similarities. Inhibition at $t = 60s$ did not produce significant differences in rebound pressing, contrary to the effects of 10s inhibition of the MD (Figure 20C). This highlights a potential dissociation between MD -> PL connections and task re-engagement during a 30s PI procedure.
Figure 20: Comparison between 15s inhibition of MD projections to PL and 10s of Total MD inactivation. (A) Comparison of the percent change in Peak time between 15s inhibition of MD-PL projections (retro) and 10s inhibition of all MD cell bodies (MD 10s) when inhibition starts at t = 0s. (B) Same as in (A) but comparing changes in peak variance when inhibition is delivered at t = 15s. (C) Comparison of rebound pressing between 15s inhibition of MD-PL projections (retro) and 10s inhibition of all MD cell bodies (MD 10s) when inhibition starts at t = 60s
4.3 Discussion

Using retrograde targeting of MD projections to the PL cortex we were only able to reproduce a single change in timing behavior, demonstrating that many of the differences in timing behavior shown in chapter 3 are unlikely to be localized solely to the MD-PL subnetwork. The work here produced a significant rightward shift in the average peak time when inhibition was delivered at \( t = 0 \)s. Interestingly, this change in session averaged data was not associated with significant differences in individual trial start or stop times. Lastly, comparisons between 15s inhibition of MD -> PL projections and 10s MD inhibition at \( t = 0 \)s and \( t = 15 \)s found their changes in session averaged timing behavior to be qualitatively similar. However, 15s inhibition of MD -> PL projections did not reproduce the significant increases in rebound activity produced with 10s inhibition of the MD.

Across all experimental conditions in chapter 2 & 3, significant differences in the session averaged data was accompanied by significant modification in individual trial behavior. Though this is not often observed, there is nothing necessitating concurrent modulation of session and individual trial data. For example, increases in individual trial variance can lead to significant increases in peak spread independent of changes in the mean start time, stop time, or duration at the level of individual trials. However, this finding does highlight the importance of examining data across multiple levels. In relation to this study, the mean time for individual trials is inherently more susceptible to be influenced by aberrant behavior on a few trials. Previous work has looked to minimize the
effects of irregular responding through eliminating trials which did not meet criteria such as starting after or ending before the target duration (Church et al., 1994; Swearingen & Buhusi, 2010). Unfortunately, in perturbation experiments, the removal of such trials may take out trials that are instantiations of the modulations true effect. Outlier detection methods based on metrics such as Z-score can be implemented to provide a systematic approach to trial removal. Setting high thresholds can minimize the chance of accidentally removing true effects.

The fact that inhibition of MD → PL projections produces qualitatively similar though quantitatively muted changes in timing does not come as a complete surprise. This has been found in other studies investigating the role of the anterior thalamic nucleus (ATn), part of the limbic thalamus, in various types of cognitive function. Behavioral effects of ATn lesions are generally more pronounced than lesions disrupting any of their individual inputs, suggesting that no single pathway supports all cognitive aspects (Aggleton, Neave, Nagle, & Hunt, 1995; Powell et al., 2017; Sziklas & Petrides, 2000; Warburton, Morgan, Baird, Muir, & Aggleton, 1999; Wright, Vann, Aggleton, & Nelson, 2015). This may be a general principle of cortico-thalamo-cortical networks. However, lack of a significant difference in rebound pressing does seem to indicate that task related information is not distributed equally across cortical areas associated with the MD. Conversely, the MD may handle retrospective and prospective temporal information differently. That is temporal relationships between events in the past,
retrospective, may be more robust against perturbation than ongoing timing of instrumental actions, prospective (MacDonald, Fortin, Sakata, & Meck, 2014).

The ability of pharmacological inhibition of the PL in rats to produce more robust effects seems to contradict this interpretation (Buhusi et al., 2018). However, it is important to note the implantation of guide cannulas over the PL cortex resulted in severe damage to the ACC and parts of the dorsal infralimbic (IL) cortex. This may make the animal more susceptible to the inhibition condition making the effects greater than they would have been in an otherwise uncompromised system. Additional work using liquid cooling of the mPFC seems to support this idea, as it was still shown to lead to delays in timing behavior, but the effect was milder than those found in the above study (Xu et al., 2014).

The work here demonstrates the resilience of timing behavior to inhibition of MD projections to the PL. Inhibition of this pathway only found a single significant change in timing behavior, producing a delay in the peak time of pressing in mice when inhibition was applied at the onset of a peak trial. Interestingly, though not significantly different from non-laser trials for all conditions, the magnitude of the differences in peak time and variance for session data was qualitatively similar to 10s of total MD inhibition. Conversely, MD ➔ PL inhibition had no effect on rebound pressing demonstrating a potential dissociation in the effects on retrospective and prospective timing.
5. Conclusion

5.1 Role of mediodorsal thalamus in proper timing behavior

As the work presented here was limited to modulation of the MD, the obvious question of how the MD contributes to the neural underpinnings of time perception. Current literature from within the field of interval timing, as well as broader works related to aspects of cognitive function point toward two potential roles of the MD: (i) supporting cortical timing signals through recurrent connections (ii) updating cortical timing signals by relaying information from motor output regions in a feedback network via the basal ganglia. The first option finds considerable evidence from computational work within the field of timing (Paton & Buonomano, 2018) and experimental work aimed at understanding working memory (Bolkan et al., 2017; Schmitt et al., 2017). The second from recent work examining the how output from the SNr influences interval timing behavior (Toda et al., 2017).

5.1.1 Mediodorsal thalamus supports temporal activity in the cortex

Though focus on the mPFC has produced great insight into its role in time perception, the mPFC does not operate in a vacuum. In rodents, the mPFC is defined largely by the existence of strong reciprocal connections with the MD (Heidbreder & Groenewegen, 2003). Similar to sensory thalamo-cortical interactions, MD afferents drive feed-forward inhibition within the mPFC (Delevich et al., 2015). While seemingly inconsequential, elevations in excitatory-inhibitory (E/I) balance within the mouse mPFC can have profound impairments
on information processing (Yizhar, Fenno, Prigge, et al., 2011). A recurrent neural network model investigating disruptions of E/I balance expands on these findings, showing rises in E/I ratio were associated with increased susceptibility to internal noise and external distraction (Murray et al., 2014). As the most parsimonious explanation for the results found in chapters 2 & 3 is tied to attention, the ability of E/I modulation to effect aspects of cognition associated with attention is a highly encouraging and a potentially fruitful avenue for future research. Additionally, patients with Schizophrenia exhibit elevated E/I ratios and have demonstrated comorbidity of MD deterioration and impairments on in interval timing tasks (Mole, Winegardner, Malley, & Fish, 2018).

Extensive computational work, based on ‘time cell’ neural signatures found in the frontal cortex, has shown this neural activity demonstrates many of the same properties found in behavioral expressions of interval timing (Hardy & Buonomano, 2018; Hardy, Goudar, Romero-Sosa, & Buonomano, 2018; Karmarkar & Buonomano, 2007; Laje & Buonomano, 2013). Canonical ‘time cell’ activity (Figure 1B) involves the sequential activation of cells within a brain region filling the duration of a to-be-timed interval. These signals, across multiple brain regions, have been shown to have a similar trait of increasing their variance the later they fire within a sequence (Bakhurin et al., 2017; MacDonald et al., 2011; Mello et al., 2015). This conforms with the scalar property found in the behavioral expression of timed motor output (Buhusi & Meck, 2005; Gibbon et al., 1984; Matell & Meck, 2000; Rakitin et al., 1998).
Remarkably, recent optogenetic work examining interactions between the MD and mPFC has established a connection between the same neural signatures and behavioral performance, in a working memory task (Bolkan, et al., 2017). Bolkan and colleagues, showed that during the delay period of a working memory task the mPFC produced the same ‘time cell’ signature found during timing tasks (Bakhurin et al., 2017). Importantly, inhibition of the MD disrupted the temporal patterning of mPFC neural activity and led to impaired performance on the working memory task providing evidence of a causal link between the neural activity and behavior.

Importantly, thalamo-cortical relays are not categorically specific, but work to sustain and coordinate task relevant cortical activity (Nakajima & Halassa, 2017). This indicates the MD is providing support for cortical processing that, while not completely task independent, is partially divorced from specific aspects of an individual task. The findings from experiments in chapter 2 align with the concept of the MD being quasi-specific. That is, while the effects between task are qualitatively similar, they differ in magnitude based on non-temporal features related to the specific timing task. In this way it can act as a general support network for cortical timing signals while its effectiveness in this role is vulnerable to extraneous factors.

5.1.2 Mediodorsal thalamus updates the cortex via connections with motor output regions

Alternatively, the MD may influence interval timing behavior through gating information through a feedback loop via the basal ganglia. This loop is relatively
unexplored in relation to time perception and can occur at various levels. An internal loop exists through interactions between the striatum and MD, in addition to an external loop connecting the MD through basal ganglia output signals. In agreement with circuitry outlined in the SBF model of timing, connections between the striatum and thalamic nuclei can form an internal timing loop providing reentrant information to the cortex during a timed interval (Yin & Troger, 2011). However, the level of information, temporal or otherwise, that this connection transmits requires greater experimentation.

The MD plays a fundamental role in the internal monitoring of movement sequences in the PFC through corollary discharge. In non-human primates performing a sequential saccade task, muscimol inactivation of the MD prevented effective compensation in the direction of a second saccade relative to the first (Sommer & Wurtz, 2002). This phenomenon is not species or sensory specific appearing in rodents (Fee, Mitra, & Kleinfeld, 1997) and songbirds (Striedter & Vu, 1998) as well. In the songbird, corollary discharge is thought to support the learning and production of precisely timed vocalizations linking this feedback signal to the field of time and time perception.

Investigation of basal ganglia output signals provides an additional feedback network by which the MD may play an important role. Optical stimulation of the GABAergic projections from the SNr to the deep layers of the superior colliculus (SC) produced delays in the timing of subsequent licking behavior (Toda et al., 2017). Tracing work has shown neurons in the deep layer of the SC to project to the MD as well as other HO thalamic nuclei in the mouse,
a network strikingly similar to the originally identified pathway responsible for corollary discharge in non-human primates (Sommer & Wurtz, 2002).

Additionally, increased inhibition resulting from MD activity can shape integration of hippocampal-evoked activity in mPFC pyramidal neurons. Extracellular recordings in anesthetized rats found MD stimulation to inhibit or facilitated PFC neuron firing evoked by fimbria/fornix (FF) stimulation depending on the temporal contingency between the activity of the three brain regions (Floresco & Grace, 2003). Therefore, the MD may play a vital role in how influencing information exchange between two regions vital for proper timing behavior. This findings also implicates a novel pathway for incorporating the hippocampus into timing networks (Yin & Troger, 2011).

The role of the MD as a component of a feedback loop is in agreement with its broad role as a site for information integration. Additionally, in this theoretical role the MD is providing unique information to time cells within the cortex, making it distinct from the aforementioned role in supporting temporal information in the cortex. However, analogous to the reciprocal connectivity characteristic of cortico-thalamic networks, the MD projects back to the striatum. These projections have a topographical organization across segments of the MD and span both the nucleus accumbens and caudate putamen in rodents (Erro, Lanciego, & Gimenez-Amaya, 2020). Therefore, the MD may play a similar supportive role in stabilizing temporal signals in the striatum as it does in the cortex rather than being a part of a cortico-striato-thalamo-cortical feedback loop.
5.1.3 Multiple roles of the mediodorsal thalamus in timing behavior

The relationship between proper MD activity and information retention across delays strongly implicates a role in interval timing behavior. However, the significance of MD-PFC interactions on interval timing is unclear. While research in this area is limited, there is both theoretical and experimental evidence implicating the MD in time perception. It has been argued that the neural processes that underlie working memory and timing are not just anatomically similar, but the same neural representations (Matell, Meck, & Lustig, 2005). Work in non-human primates has found deficits in motor-timing after local infusions of muscimol into mPFC projecting thalamic neurons (Wang, Narain, Hosseini, & Jazayeri, 2018). Moreover, lesions of the MD in rats produced deficits in both the acquisition and expression of timed action responses (Yu, Gupta, & Yin, 2010). Importantly, Yu and colleagues, (2010) found deficits when the duration between subsequent responses was in the 10s of seconds range, dissociating the MD from purely motor-timing.

It is worth mentioning that these potential roles are not mutually exclusive. As shown in chapter 4, modulation of an individual area connected with the MD can reproduce some effects observed with total inhibition, such as rightward shifts with inhibition at t = 0s, with without reproducing others (i.e. rebound pressing). Interestingly, tracing studies have demonstrated parallel thalamo-cortical inputs between the MD and mPFC. Using retrograde tracers, researchers have shown projections from the medial and lateral regions of the MD follow a dorsoventral topology within the mPFC in rats, respectively (Alcaraz, Marchand,
Courtand, Coutureau, & Wolff, 2016). Specifically, the lateral portions of the MD innervate the dorsal PL as well as cingulate cortex, while the medial portions of the MD innervate the ventral PL and IL.

Additionally, subcortical afferents of the MD also contain a mediolateral topography (Groenewegen, 1988). Feedback from motor output regions such as the SNr and GP innervate more lateral regions, while neuromodulatory input from dopaminergic regions such as the VTA and SNc project more medially (Figure 21). The integration of these distinct subcortical information with ongoing cortico-thalamo-cortical signaling may indicate dissociable roles for these MD subnetworks. Therefore, variations in the ventrodorsal thalamo-cortical connectivity within the mPFC and/or mediolateral variations between subcortical afferents to the MD may account for these differential roles and explain the results shown in chapter 4.
Figure 21: Schematic of networks associated with the MD  The reciprocal cortical (top) and subcortical (bottom) connection between the medial (green & cyan), central (yellow), lateral (orange), and paralamellar (red) segments of the MD based on tracing from (Groenewegen, 1988). Cortical connections include the secondary motor (M2), anterior cingulate (ACC), orbital frontal (OFC), prelimbic (PL), and infralimbic (IL). Subcortical afferents are from substantia nigra paras reticulata (SNr) and compacta (SNC), superior colliculus (SC), globus pallidus (GP), ventral tegmental area (VTA), amygdala (AMG) and lateral entorhinal cortex (LEC)
It is important to note that the work in chapter 4 was focused more heavily on the medial MD \(\rightarrow\) PL connections. This is also the region of the MD connected to dopaminergic networks which have been strongly implicated in modulating ‘clock speed’ effects. This may be one possible explanation for the current findings. While there is compelling evidence for the role of potential MD subnetworks in mediating distinct aspects of timing behavior, systematic testing of individual subnetworks is important for fully understanding the contributions of the MD as both a relay and supporter of temporal information.

**5.2 Integrating the mediodorsal thalamus into timing theories**

**5.2.1 Pacemaker-accumulator models**

Many of the most influential theories of interval timing such as the scalar expectancy theory (SET) are members of a ‘pacemaker-accumulator’ (PA) family of timing models (Simen, Rivest, Ludvig, Balci, & Killeen, 2013; Treisman, 1963). Within this framework (Figure 22A) pulses from a pacemaker are accumulated downstream and compared to a known duration in reference memory. When the ratio between the current ‘clock reading’ and reference reaches a threshold, temporally guided behavior is generated (R. M. Church et al., 1994). Since its conception, substantial work has been aimed at assigning components of the PA model (e.g. pacemaker, accumulator, reference memory) to particular brain regions based on experimental findings related to timing as well as cognition broadly.
For example, evidence from timing research (Meck et al., 2013; Meck et al., 1984) implicates the hippocampus as the neural analog to the reference memory stage. Additionally, the cortex has been implicated as the brain's equivalent to the pacemaker’s (Binkofski & Block, 1996). However, how exactly these two regions interact during the comparison phase is still poorly understood. With the MD being capable in gating interactions between these two regions (Floresco & Grace, 2003) the MD may be the instantiation of the PA comparator phase.

Additionally, the rate at which temporal information is accumulated depends on the pacemaker rate alongside the activation of an attentional ‘switch’. Though considerable work has shown that cortical dopamine can influence the speed of the pacemaker (Buhusi, 2003; Cheng, Ali, & Meck, 2007; Malapani et al., 1998; Matell & Meck, 2004; Meck, 2006; Meck, 2006), the neural underpinning responsible for the attentional ‘switch’ is more speculative (Lejeune, 2000). The electrophysiological evidence from working memory tasks (Bolkan et al., 2017; Schmitt et al., 2017) demonstrate the MD to have characteristics indicative of the attentional ‘switch’ through amplifying time relevant signals.

The integrative nature of the MD makes it well-suited for connecting the various components within PA models of timing. Though many of the components have identified neurological counterparts such as the cortex, hippocampus, and striatum, gaps remain as to what brain regions contribute to their interactions. As outlined the MD may potentially play multiple roles as a comparator or switch mechanism, through gating the interactions between the
cortex and hippocampus or amplifying cortical representations of time, respectively.

5.2.2 Striatal-beat frequency (SBF) model

The striatal-beat frequency (SBF) model has found experimental (Matell & Meck, 2004) as well as computational support (Sorinel A Oprisan & Buhusi, 2011) as a biologically plausible model of timing. Within the SBF framework, oscillatory neurons in the cortex are synchronized by dopaminergic inputs from the ventral tegmental area (VTA) at the start of a duration and begin oscillating at their endogenous frequencies. When a reward is given at the end of a to-be-timed duration, dopaminergic neurons in the substantia nigra pars compacta (SNc) fire, modulating plasticity of cortico-striatal synapses based on the coincident pattern of cortical firing.

Unlike the traditional PA model, the SBF does make explicit assumptions as to the role of the thalamus (Figure 22B). The SBF posits that the thalamus participates in a feedback loop updating/gating temporal information back to the cortex and striatum. This function, while in agreement with the behavior findings in chapters 2, 3, & 4, does not capture the potential role of the MD in supporting cortical oscillatory behavior and is vaguely similar to the possible involvement of the MD at the comparator stage in PA models.

Additionally, as with many models of timing, the SBF looks at the thalamus as a singular entity rather than a collection of distinct nuclei. Along with the MD, other HO nuclei such as the paraventricular (PV) nucleus project predominantly to the frontal cortex including the heavily discussed IL and PL sub regions.
(Vertes & Hoover, 2008). Moreover, posterior regions of the PV project to the
dorsal striatum, the region most strongly associated with ‘time cells’ within the
basal ganglia (see Lusk, Petter, MacDonald, & Meck, 2016). Therefore, while the
work presented here is aimed at understanding how the MD fits into current
models of interval timing it is worth noting there are many additional thalamic
nuclei worth investigating.
Figure 22: Models of interval timing. (A) Schematic of the components of pacemaker-accumulator model adapted from (Taatgen, van Rijn, & Anderson, 2007). (B) Striatal-beat frequency model with associated brain regions adapted from (Gu, van Rijn, & Meck, 2015). (C) Example of a neural trajectory from state-dependent theory of timing adapted from (Karmarker & Buonomano, 2007).
Updating the SBF with the inclusion of recurrent connections between cortical oscillators and the thalamus would provide two major benefits over its current form. First, it would provide a better match to the anatomical substrates it is looking to model, and second, it would allow for a mechanism by which to test how attention to timing may influence timing within the system. In addition, including the proper dopaminergic innervation to thalamic nuclei such as the MD from the VTA as well as SNc would allow for investigation of a potential secondary network associated with ‘clock speed’ effects.

5.2.3 Models of timing as an inherent neural computation

While most models have been designed with the explicit purpose of encoding time, recent theories have looked to do away with the entire idea of a dedicated timing network. Based on the extensive number of brain regions associated with interval timing, and the heavy overlap between time perception and other cognitive functions, many researchers have proposed that timing is an inherent neural computation (Hass & Durstewitz, 2016). This idea has been spurred by experimental work showing time-representing mechanisms being active even when timing is not explicitly required.

Recent analysis of neural activity in the prefrontal cortex found elapsed time explained a substantial proportion of variability in the firing rates across multiple tasks in various environmental conditions although timing was not a critical component of the tasks (Hyman, Ma, Balaguer-Ballester, Durstewitz, & Seamans, 2012). This same property has been demonstrated in the
hippocampus, where ‘time cell’ activity can be seen as a byproduct of motion (MacDonald et al., 2011) or memory recall (MacDonald et al., 2013).

This idea is at the heart of state-dependent timing models (Figure 22C) (Karmarkar & Buonomano, 2007; Laje & Buonomano, 2013). As timing is so fundamental to all neurological processes, such as behavioral planning and working memory, an organism’s perception of time is built into the neural dynamics in the form of time-varying signals. These models of time perception beg the question as to how a system of distributed and independent timing mechanisms form a unified concept of time with time. It is still believed that these distinct representations of interval time would have to be joined at a ‘temporal hub’ similar to that found in the SBF.

Integrating the MD into state-dependent models of timing is quite simple in that it will play the same role as it does in other forms of cognition. Despite this, recurrent neural network models exploring state-dependent neural trajectories have not yet begun integrating thalamic contributions into their models. The thalamus has a particular connectivity motif, lacking the extensive excitatory recurrence commonly found in the cortex. This architectural distinction likely alters the computational role of the thalamus in relation to that of the cortex, thus modifying the computational abilities of networks that include its unique connectivity. As many timing networks relying solely on cortical based connectivity rules have trouble timing longer durations, the addition of diverse connectivity rules may provide one solution for overcoming current limitations in biologically based models of timing behavior (Lusk, 2019).
5.3 Further Directions

Many questions still remain in relation to the role of the MD in time perception. The experiments performed in chapters 2, 3 & 4 focused on changes in timing behavior in response to perturbations of MD activity. Therefore, it is still unclear the exact electrophysiological signals underpinning interval timing within the MD and its associated networks. Drawing from work in other fields, evidence suggests that activity within the MD as well as its cortical targets produce ‘time cell’ activity patterns with individual cells exhibiting sequential bouts of activation across an interval. However, still to be demonstrated is whether these cells show characteristics such as temporal scaling like ‘time cells’ from other brain regions (Mello et al., 2015; Wang et al., 2018).

There is evidence indicating that the MD may not have ‘time cell’ activity like those identified in the cortex, striatum, and hippocampus. Recordings from frontal cortex projecting thalamic neurons in non-human primates during a sub-second timing task revealed far lower levels of temporal scaling in the thalamic neurons relative to cortical and striatal cells (Wang et al., 2018). While scaling was not absent in these cells, researchers concluded that scaling found in connected regions was not likely to be a product of thalamic activity. However, this study relied on sub-second durations and experiments have identified the importance of MD influence on cortical activity increases over longer delays. This makes its potential role in timing supra-second durations distinct from sub-second durations. Additionally, concurrent recording and perturbation techniques
can help establish causal roles between neural activity and the behavioral findings in the current work.

How the MD’s effects on interval timing behavior are localized across its multiple subnetworks is still unclear. The findings in chapter 4 point to the idea that different cortical regions connected to the MD may contribute to distinct aspects of timing behavior. Inhibition of PL projecting MD neurons reproduced some of the behavior changes from total inhibition, but not others. Systematic testing of the connections between regions of the frontal cortex would provide significant insight into how timing behavior is distributed across the cortex. Additionally, comparing the effects of thalamo-cortical and thalamo-striatal could be a fruitful avenue for future research.

Lastly, additional behavioral testing is still needed to fully grasp the contributions of the MD to time perception. While the results here point to the role of the MD in modulating attentional processes related to timing, psychometric modulation of timing signals is still necessary. Previous work has shown increases in timing deficits when attentional processes are taxed through decreasing the salience to timing cues. Inhibiting the MD under various levels of cue salience can validate the theoretical role of the MD outlined here. Alternatively, the use of PI procedures with gaps in the timing signal have shown dissociable effects between hippocampal and cortical lesioned animals (Meck, 1996), with hippocampal lesioned animals displaying an amnesia of the cue prior to the gap. This is similar to the rebound pressing exhibited in chapter 3.
Moreover, a hallmark of interval timing is the constant ratio of timing precision relative to the duration being timed (i.e. scalar property). How inhibition of a brain region effects the scalar property provides exceptional insight into how it contributes to time perception. The work in chapter 3 provides insight into how the length of optical inhibition leads to proportional changes in timing behavior. However, direct testing of MD inhibition and the scalar property are still needed. While the work presented here provides strong evidence for the role of the MD in interval timing behavior, better understanding at the anatomical, electrophysiological, and behavioral level is still needed.
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Biography

Nicholas Adam Lusk was born in Michigan, USA on February 25, 1989. He attended the University of Michigan in Ann Arbor, Michigan, USA and received his Bachelor of Sciences degree in Psychology in May 2011. He attended Duke University in Durham, North Carolina, USA and received his Doctor of Philosophy in Psychology and Neuroscience in May 2020.

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