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An Optogenetic Investigation into the Roles of Striatum, Dopamine, and Collateral Behavior During Interval Timing

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by

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EPIGRAPH

Where is it, this present? It has melted in our grasp, fled
ere we could touch it, gone in the instant of becoming.

William James

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LIST OF ABBREVIATIONS

AAV	adeno-associated virus
BeT	Behavioral Theory of Timing Model
ChR2	channelrhodopsin 2
CV	coefficient of variation
DA	dopamine
DAT	dopamine transporter
DS	dorsal striatum
fMRI	functional magnetic resonance imaging
GABA	γ-aminobutyric acid
GPi	internal globus pallidus
LeT	Learning to Time Model
lPGi	lateral paragigantocellular nucleus
PD	parkinson's disease
PETH	peri-event time histogram
RTCPP	real-time place preference
SMA	supplementary motor area
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SPN	spiny projection neuron
vGAT	vesicular glutamate transporter

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ABSTRACT OF THE DISSERTATION

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Time is a fundamental dimension of decision-making. *When* to perform an action is often as important as what action to take, and thus our sense of time is essential for guiding everyday behavior. Our perception of time allows us to form temporal associations between events and to detect irregularities in our environment; it allows us to optimize our choices and anticipate future consequences. However, unlike other sensory modalities, our sense of time appears to be entirely self-generated, as if our brain possesses—as some researchers have supposed—an "internal clock." We clearly have a sense of time, yet how time is tracked and encoded by the brain remains mysterious. Animal behavior demonstrates features of timing across many timescales, from milliseconds to months. This dissertation focuses on timing on the scale of seconds-to-minutes, most commonly known as interval timing. Basal ganglia circuits are essential for motor control, action selection, and reinforcement learning. However, numerous lines of evidence also suggest that the basal ganglia –in particular the dorsal striatum and nigrostriatal dopamine – play a central role in interval timing. Does the basal ganglia serve a motor-independent function during interval timing, or is its role in timing fundamentally intertwined with action? In animal models of interval timing, researchers widely observe the development of non-instrumental, collateral behaviors as animals learn to time under operant conditioning. This dissertation explores the idea that these collateral behaviors can act as a functional mechanism to support timekeeping. We suggest that the basal ganglia's role in interval timing may indeed be tied to these collateral behaviors. Chapter 1 reviews modern conceptions of how interval timing can be categorized, as well as the lines of evidence suggesting a role for motor-related brain regions in interval timing. In Chapter 2, I discuss the peak-interval operant task used throughout our experiments, I provide an analysis of the emergent behavior, and finally suggest a metric by which to assess timing accuracy on a trial-by-trial basis. In Chapter 3, I describe our optogenetic

methodology for disrupting collateral behavior by disrupting striatal activity, and I demonstrate how these behavioral disruptions subsequently affect temporal decisions. In Chapter 4, I disrupt collateral pressing behavior by optogenetically manipulating a brainstem motor output nucleus. I demonstrate that the effects of disrupting action in the brainstem result in similar timing deficits as does disrupting action via the dorsal striatum. In Chapter 5, I assess how stimulating dopamine neurons in the SNc affects timing performance, and validate our methodology and nigrostriatal dopamine release using fast-scan cyclic voltammetry. I demonstrate that dopaminergic stimulation in these experiments only affects timing behavior insofar as collateral pressing behavior is also disrupted.

Chapter 1: Introduction and Background

1.1 *Interval Timing and Adaptive Behavior*

Adaptive behavior is organized around a central truth: actions have consequences, and some consequences are better than others. Animals have the extraordinary capacity to learn from the outcomes of their behaviors; they can refine their abilities, anticipate future events, and optimize their choices. One vital component of adaptive behavior is the ability to track the passage of time. Indeed, *when* to perform an action is often as critical as what action to take. This sense of time guides actions and informs decision-making. It is malleable, yet can be refined into exquisite accuracy. Although time is a central dimension of animal behavior, how the brain encodes and tracks intervals of time remains mysterious.

Of course, behavioral timing is a very broad topic; the brain guides behavior across timescales of vastly different magnitudes. Complex motor coordination requires precise timing on the scale of milliseconds, steering behaviors like speech, musical production, and athletics. On the other hand, 24-hour circadian timing is essential for sleep-wake cycles, appetite/food-seeking, and other daily rhythms. Between these extremes of behavioral timing is interval timing, which is considered to take place on the order of seconds-to-minutes. It is this order of timing that is the focus of this dissertation.

Interval timing, like the traditional senses, provides information. It allows us to know when something is taking too long or happening too quickly, or when the right moment to perform or not perform an action is. Our environment is abound with temporal regularities, whether they come from other animals or from the physical environment itself. Interval timing allows animals to learn from, adapt to, or even exploit these regularities. For example, when to move on when foraging, when to initiate chase, when to reemerge from hiding.

Duration, like distance, is a measure of relativity. Distance measures relative space and duration measures relative time. And just as animals can compare distances, animals too can compare durations. Humans have the benefit of an arsenal of quantified, labeled time: we can all approximate 3 seconds or 30 seconds, 2 minutes or 20. Although animals do not have these numerical references, they do have references built upon learned experiences. The guiding question of this dissertation is: how do brain and behavior encode and measure intervals of time. In addressing this question, I'll present evidence arguing that one important mechanism of interval timing critically involves learned motor behavior. In this introduction, I review research on the theories and neuroanatomical correlates of interval timing that form the foundation of my own work.

1.2 *Taxonomies of Interval Timing*

Time can be a difficult concept to wrap one's head around. Interval timing, as it exists in the realm of animal behavior, is no exception. Interval timing is involved in a number of different behaviors, each likely involving different combinations of neural systems and processes (Coull and Nobre, 2008; Paton and Buonomano, 2018). To give structure to discussions about interval timing, it has proven useful to construct a taxonomy of interval timing.

1.2.1 *Retrospective vs Prospective Interval Timing*

In some taxonomies, interval timing is separated into retrospective and prospective interval timing. In this framework, retrospective timing involves an estimation of the duration of an elapsed interval that one did not know to attend to (Rijn, 2018). For example, at the end of a short interaction, one might ask, "How long were we talking?" In retrospective timing, the start and stop of an interval are determined after the fact, and therefore interval timing of this form is

not directly used to guide the timing of actions or responses.

In prospective timing, the start of the to-be-estimated interval is known beforehand, or before the interval is completed, and the passage of time can therefore be used to guide action or response timing. For example, following an alarm call (start signal), a rodent may burrow into hiding for a learned/optimized duration of time. Most interval timing research focuses on prospective timing, where a human/animal is aware through instruction or procedural training that the task requires them to time, and this timing is then used to guide decision making.

1.2.2 *Implicit vs Explicit Interval Timing*

Another important taxonomy of timing suggests that interval timing can be divided into implicit and explicit timing. In this division, explicit timing refers to deliberate, conscious estimation or reproduction of duration. In this mode of timing, accurate timing itself is the task goal, and the discriminated or reproduced interval is compared to a learned reference (Coull and Nobre, 2008). This could occur when reporting whether one presented tone is longer or shorter than another, or when one must make a motor response of a particular duration (pressing a key for 10 s, or pressing a key every 1 s). Implicit timing, on the other hand, occurs when timing itself is not the task goal but still guides actions, expectations, or decisions – even if unconsciously. For instance, when waiting at a red light, implicit timing informs heightening expectations that the light will soon turn green. Or, when foraging, implicit interval timing informs decisions about when to move on from a given spot. In the real world, individual behaviors can include elements of both explicit and implicit timing to different degrees, and are further shaped by factors known to alter one's perception or estimation of time. These include factors like attention, emotion, or even sensory context (Coull et al., 2004; Droit-volet, 2018).

Despite these real-world complexities, the distinction between explicit and implicit timing has given researchers a framework for exploring how distinct neural processes may be differently involved in various modes of interval timing.

1.2.3 *Sensory/Perceptual Timing vs. Motor Timing*

A final distinction commonly made within discussions of interval timing is the distinction between sensory and motor timing (Paton and Buonomano, 2018). Sensory (or perceptual) timing refers to timing where the duration of or between external sensory stimuli provides the temporal information guiding behavior. In essence, animals are timing the duration of an exogenous sensory cue, and that information in turn guides a choice or motor response. For example, in the lab, a simple sensory timing task involves comparing the durations of lights or tones. In the real world, sensory timing is used to extract information from the temporal evolution of another animals' behavior or from regularities of the physical environment. It is important to note however, that sensory timing often involves empty intervals, where the absence of changing sensory information *is* the exogenous sensory information. For instance, one can think back to the example of waiting at a red light. In later chapters of this dissertation, I argue that during such empty intervals (among other situations), animals can implicitly learn to use their own motor behavior to more accurately discriminate the duration of such intervals. This strategy, however, is different than what is commonly referred to as motor timing.

Motor timing refers to the ability to produce behaviors that themselves have a precise temporal structure. This can, for example, be in the form of a sustained, periodic, or temporally delayed action. Rhythmic or repetitive tapping is a common example of motor timing. Outside of the lab, motor timing is often learned or optimized according to a desired outcome. For example,

predators might learn an optimal duration for constricting prey of different sizes in order to ensure death.

It doesn't take long for one to realize that some, if not all, examples of interval timing include components of both sensory and motor timing. Activity correlated with the expectation of sensory events has been observed in primary visual cortices following training in sensory timing tasks (Hussain Shuler, 2016). And indeed, recent work from our own lab suggests that sensory feedback from motor behavior is critical to accurate motor timing. Like with other distinctions within interval timing, it has still been useful to distinguish between behaviors relying primarily on sensory versus motor timing for organizing discussions about the different neuroanatomical correlates of interval timing, which we will talk about later on.

1.3 *Evidence for Interval Timing within Motor Control Circuits*

Any time spent reviewing the body literature exploring the neuroanatomical correlates of interval timing will reveal a common theme: circuits typically associated with action and motor function appear to play a central role (Coull and Droit-volet, 2018; Coull et al., 2011; Gouvea et al., 2015; Merchant and Yarrow, 2016; Rao et al., 2001). Of these neuroanatomical correlates, the basal ganglia (BG) and related dopaminergic systems are perhaps the most widely implicated. The following sections review BG architecture and the role of dopamine (DA) in movement and action.

1.3.1 *Basal Ganglia*

The basal ganglia are a group of interconnected subcortical nuclei essential for transforming and refining cortical activity into directed behavior (Dudman and Gerfen, 2015). The basal ganglia are thought to be central to action selection, voluntary motor control,

reinforcement and procedural learning, in addition to less well-understood aspects of cognition and emotion. These broad functions of BG are unsurprising given its input-output architecture. The primary input nucleus of the basal ganglia, the striatum, receives convergent excitatory input from virtually all regions of the cerebral cortex. The output nuclei of the basal ganglia project both to behavior-effector systems as well as back to the cerebral cortex, forming distinctive, parallel corticostriatal loops (Alexander, 1986).

Roughly 95% of neurons in the striatum are GABAergic projection neurons known as spiny projection neurons (SPNs). Modulating the activity of these SPNs is broad dopaminergic input from midbrain dopamine neurons in substantia nigra pars compacta (SNc) (Dudman and Gerfen, 2015). This dopaminergic modulation is a critical component of reinforcement learning. As is apparent in Parkinson's disease (PD), where dopaminergic neurons of the SNc progressively degenerate, this dopaminergic modulation is also essential for action selection and voluntary motor control (Gerfen and Surmeier, 2011; Howard et al., 2017; Morris et al., 2006; Redgrave et al., 1999).

Striatal SPNs are classified by the particular subtype of dopamine receptor that they express. SPNs expressing the D1-type dopamine receptor form the canonical 'direct' pathway to the primary basal ganglia output nuclei, the substantia nigra pars reticulata (SNr) and internal globus pallidus (GPi). SPNs expressing the D2-type dopamine receptor form the 'indirect' pathway, projecting indirectly to SNr and GPi via the external globus pallidus and subthalamic nucleus. D1- and D2-type SPNs were traditionally proposed to have opposing influence on motor control (Albin et al., 1989). Although evidence of this dichotomous relationship indeed exists (Kravitz et al., 2010), more recent evidence suggests that these pathways have more nuanced, intertwined effects on motor behavior (Picconi et al., 2014).

1.3.2 *Midbrain Dopamine and Movement*

Dopaminergic neurons of the SNc are best known for their role in Parkinson's disease. In PD, progressive degeneration of these neurons leads to profound deficits in motor control. Among other symptoms, PD patients display slowed and reduced movement, tremor, limb rigidity, and difficulty initiation action (Jankovic, 2008). These symptoms worsen as SNc degeneration progresses, and the most common line of treatment for PD is dopamine therapy. The pathophysiology of PD makes it clear that dopamine plays a crucial role in motor control. The neuroanatomical connectivity of dopamine neurons substantiates this further: as discussed in the previous section, a primary target of SNc dopamine neurons is the striatum, a structure known to be central to action learning and selection (Gerfen and Surmeier, 2011; Howard et al., 2017; Morris et al., 2006; Redgrave et al., 1999). Much of the literature on dopamine's role in action has focused on the reinforcement signals it provides, like the seminal experiments on reward prediction error (Schultz, 1997). However, recent evidence has suggested a more direct role for nigral dopamine in movement.

A body of evidence now suggests that dopamine plays a significant role in movement vigor (Mazzoni et al., 2007; Panigrahi et al., 2015; Da Silva et al., 2018). Parkinson's patients show reduced movement vigor (Mazzoni et al., 2007). Rodent models of PD show a reduced movement vigor that is restored upon dopamine repletion, and electrophysiological representation of movement vigor striatum has been shown to be dependent on dopamine (Panigrahi et al., 2015). Additionally, researchers have identified dopamine neurons in SNc that increase their activity prior to self-paced movement in a manner that is not action-specific but is related to the vigor of movement (Da Silva et al., 2018). Optogenetic stimulation of these neurons in immobile mice increased the probability and vigor of subsequent movement. Related

to movement vigor, some evidence suggests that SNc dopamine neurons represent directional, vector components of movement velocity and acceleration (Barter et al., 2015). From PD symptoms to electrophysiological recordings, it has become clear that SNc dopamine plays a significant but not fully understood role in motoric components of action. Subsequent chapters explore the relationship between dopamine and interval timing, as well as movement in interval timing. In the original experiments presented in this dissertation, we explore the idea that the effect of dopaminergic manipulation on interval timing could in part result from the significant role dopamine plays in movement itself. In the following subsections, I review the body of research on dopamine, the basal ganglia, and cortical motor areas in interval timing.

1.3.3 *Disease States*

Impaired interval timing has been described in a number of neurological disease states involving motor control and the basal ganglia. In Parkinson's disease, dopamine neurons in the SNc progressively degenerate, severely disturbing normal nigrostriatal dopaminergic modulation of striatal SPNs. PD patients have been shown to have deficits in encoding and reproducing intervals of time, as well as impaired perceptual estimation of time intervals (Honma et al., 2016; Malapani et al., 1998, 2002; Pastor et al., 1992). These deficits in explicit duration estimation are improved by dopaminergic treatment (Malapani et al., 1998). It should be noted that PD does not lead to a universal under- or overestimation of interval duration. When reproducing two previously learned intervals, one short and one long, PD patients overestimate the short interval and underestimate the long interval, resulting in a so called 'migration effect' (Malapani et al., 1998). As I discuss in the next chapter, pharmacological and optogenetic studies have also implicated dopamine in interval timing. The migration effect seen in PD patients, however, suggests a more complicated effect of PD on interval timing than just a slowing down or

speeding up of time perception. In later chapters, I propose a framework for reconciling this bidirectional effect on DA depletion in PD.

Although motor deficits are characteristic of PD, the evidence of *motor timing* deficits in PD is conflicting (Coull et al., 2011), with a number of studies showing no impairment (Ivry and Keele, 1989; Spencer and Ivry, 2005). However, patients with Huntington's disease (HD), also affecting BG functioning, have been reported to show aberrant motor timing (Freeman, 1996; Rowe et al., 2010), as have patients with Tourette's syndrome (Vicario et al., 2010). Although the neurobiology of Tourette's syndrome remains mysterious, it is thought to involve abnormal corticostriatal circuitry and dopaminergic modulation (Swain et al., 2007).

Diseases involving BG appear to be accompanied by changes in interval timing. These diseases all affect the neurocircuitry and neurochemistry of the basal ganglia, and as I discuss in the next section, direct experimental pharmacological manipulations acting on BG systems have provided a notable foundation linking BG to interval timing.

1.3.4 *Pharmacology*

Some of the earliest work investigating the neurobiological mechanisms of interval timing focused on experimental pharmacological manipulations of rodents trained in interval timing tasks. In one such task, known as the interval bisection procedure, researchers train rodents to discriminate between long and short duration auditory stimuli. Following a short duration stimuli, a response on a left lever is rewarded, and following a long duration stimuli, a response on a right lever is rewarded. Responses to intermediate duration stimuli help create a psychometric function of what is commonly thought to represent duration discrimination. Interestingly, methamphetamine administration results in a leftward shift of this psychometric

function, and haloperidol administration results in a rightward shift (Maricq and Church, 1983). Methamphetamine enhances dopamine release throughout the brain, and haloperidol is an antagonist of certain dopamine receptors. This bidirectional effect of a dopamine enhancer and blocker led to the suggestion that dopamine is involved in regulating the speed of a supposed "internal clock" (Maricq and Church, 1983; Meck, 2006). The bidirectional effects on timing response functions following methamphetamine and haloperidol was shown to manifest in another common timing task called the peak procedure, a task discussed in detail in Chapter 2 (Buhusi and Meck, 2002). Subsequent pharmacological studies using drugs highly selective for D1- or D2-type dopamine receptors further suggested a role for dopamine in interval timing (Cheung et al., 2006, 2007b, 2007a). Dopamine receptors are present throughout the brain, and only recently have such drugs been targeted directly to the striatum of mice, rather than through systemic administration (Corte et al., 2019). In the peak procedure task, where animals must judge when to start and when to stop responding on unreinforced probe trials of the task, D2-receptor blockade delayed both the start and stop of responding in dorsomedial and dorsolateral striatum. D1 blockade in dorsomedial striatum delayed stop but not start times, and had no effect in dorsolateral striatum (Corte et al., 2019). It remains uncertain, however, whether these changes in decision thresholds are a result of aberrant timing or another element of striatal function.

Dopamine, of course, plays a significant role in cognition, emotion, attention, and action (Nieoullon, 2002). A natural difficulty with pharmacological investigations of dopamine in timing is determining whether the observed behavioral effects are due to a fundamental role for dopamine in timing, or whether such observed behavioral effects indirectly arise via changes in attention, emotion, movement, etc. Indeed, some researchers have directly attributed changes in

timing performance following dopaminergic modulation to changes in impulsivity, cognition, motivation, emotion and attention (Lake, 2016; Lake and Meck, 2013; Taylor et al., 2007; Ward et al., 2009). Later in this introduction, and again in Chapter 5, I discuss how changes in movement and motor control as a result of dopaminergic modification may additionally lead to changes in timing performance.

1.3.5 *Functional Magnetic Resonance Imagery (fMRI)*

An important source of evidence for motor circuit involvement in interval timing – especially in humans – comes from studies using functional magnetic resonance imaging (fMRI). fMRI detects regional changes in blood flow in the brain, and these hemodynamic changes are thought to correlate with regional changes in neuronal activation. In this section, I will use the term 'activation' to refer to positive changes in fMRI signals.

In perceptual duration discrimination tasks with humans, numerous studies using fMRI have demonstrated activation of brain circuits known to be involved in movement and action. Of prominent presence in this literature is the striatum, the input nucleus of BG, and also the supplementary motor area (SMA) (Coull et al., 2008, 2016; Hinton and Meck, 2004; Rao et al., 2001). In humans, SMA is located in the dorsomedial frontal cortex and is involved in a variety of motor-related functions, including voluntary movement, movement sequencing, action monitoring, and response inhibition (Coull et al., 2016). SMA is anatomically and functionally divided into the more rostral preSMA and the more caudal SMA proper (Schwartze et al., 2012). preSMA is preferentially recruited during non-motor perceptual timing tasks, and SMA proper shows greater activation during motor timing (Coull et al., 2016; Schwartze et al., 2012).

Exactly what role these motor-related regions play in interval timing is an active area of

research. As mentioned previously, all temporal estimations are necessarily made relative to another. For humans, this estimation can be relative to a learned, labelled duration of time (e.g 10 s) or to an unlabeled duration like a previously presented interval. Rapid, time-locked fMRI has provided some insight into evolution of brain activation during trials of perceptual duration discrimination, where subjects are asked to compare the duration of two presented visual stimuli (Coull et al., 2008). Basal ganglia and SMA activation are observed during the first stimulus – the "encoding" phase, but only SMA activation is observed during the second stimulus – the "comparative" phase. Importantly, these activations are relative to color-comparison control tasks matched in difficulty and motor requirements, where subjects are asked to compare color but not duration.

When considering evidence of human fMRI studies of interval timing, is important to note that a variety of timing tasks have been used to explore brain activity during timing, each often using different temporal durations of hundreds of milliseconds to a few seconds (Wiener et al., 2010). Interval timing is considered to extend beyond these durations, however, so it is unclear to how and to what degree the aforementioned circuits contribute to longer supra-second interval timing in humans. As we will discuss in the next section, longer supra-second intervals have primarily been studied in rodents. Still, the studies mentioned in this section make clear that circuits traditionally associated with motor-control appear to play a significant role in interval timing.

1.3.6 *Electrophysiology*

Finding electrophysiological correlates of "pure" abstract timing in the brain has been called the holy grail of interval timing research (Matell, 2014). This idea of pure, abstract timing

refers to neural processes involved in timing that are not multiplexed with other neural functions like movement, attention, working memory, etc. In simpler terms, this holy grail refers to the idea that there exists a region or computational process that serves as a discrete internal clock in the brain used to track intervals of time, similar to the superchiasmatic nucleus's role in circadian timing (Roberts, 1981). In part due to the evidence discussed in this section, however, the existence of a discrete internal clock has been supplanted by the conclusion that a number of distributed and diverse processes support the broad functions of timing (Paton and Buonomano, 2018). In primates and rodents, electrophysiological evidence has been taken to suggest that some of these processes are manifest in regions traditionally associated with movement and action control.

Using a task similar to the one discussed in detail in Chapter 2, Matell et al. (2003) recorded from ensembles of striatal neurons in rats trained to nose-poke for food at either 10 or 40 s following a cue. With the hypothesis that some action-independent timing process is occurring in the striatum, it was important to distinguish between neural activity corresponding to action and activity corresponding to timing. This was done by analyzing the behavioral and neural activity in conjunction, determining whether any neurons demonstrated time-dependent modulation of firing rate while also being insensitive to the rats' behavior. 22% of analyzed striatal neurons demonstrated a firing rate that peaked at different amplitudes at the two intervals, despite the rate of nose-poking being equivalent at those times. Although this was interpreted at the time to demonstrate time-sensitive neurons (Matell et al., 2003), later interpretations by the author of possible coding schemes represented by such neuronal activity make it less clear what information such activity represents (Matell, 2014). For example, the different amplitudes at the two intervals could be a result of the neuron being more tuned for effecting motor behavior at

one interval versus the other. Thus, it is ambiguous as to whether those neurons represent times (temporal coding) or the motor action at those times (motor coding). The impetus for this reinterpretation was in part due to a clever study using concurrent variable- and fixed-interval reinforcement schedules to control for movement. This study found that 91% of striatal cells that modulated their activity with time were also modulated by overt motor responses (Portugal et al., 2011). Thus, temporal information represented in the striatum appears to be fundamentally intertwined with activity related to context and motor function.

The idea that temporal information in dorsal striatum is multiplexed with action has been further demonstrated by recording from striatal neurons during a serial fixed-interval task where, within single sessions, rats were exposed to blocks of different fixed interval schedules with different criterion times (Mello et al., 2015). Researchers found that a significant proportion of recorded cells fired in identical ordinal positions relative to one another in time, and that the rates and duration of firing scaled with the different FI criterion times. Although many of the recorded neurons were modulated by lever pressing, this modulation was dependent upon whether when in relative time the same motor behavior occurred. So again, while it is unlikely that the striatal neurons represent pure, abstract timing, neurons do appear to signal temporal information that is not entirely explained by motor behavior (Mello et al., 2015; Motanis and Buonomano, 2015). In a similar vein, striatal population dynamics during a duration discrimination choice task have been argued to be able to predict choice better than ongoing behavior as measured through video analysis (Gouvea et al., 2015). However, as discussed in detail the next section, animals do appear to take behavioral approaches to solve timing tasks (Gouvêa et al., 2014; Killeen and Fetterman, 1988; Matell, 2014), and perhaps the multiplexing of timing and action functions of striatal neurons reflects this.

Electrophysiological studies of interval timing have also been performed in primates. One such study provided a visual cue to monkeys that specified one of three minimum durations (2 s, 4 s, and 8 s) that monkeys were required to hold a key in order to receive a reward (Mita et al., 2009). Cleverly, the hold duration specified by the visual cue was reversed during certain blocks of trials, in order to distinguish between information representing cue color and specified hold duration. Researchers examined neural activity in SMA and preSMA during the task, and found that many SMA and preSMA neurons were characterized by exponential buildup or decay that changed sharply upon movement onset. These neurons could further be distinguished between "time-graded" and "time-specific" neurons. Time-graded neurons showed similar patterns of activity during all hold intervals but had amplitudes that scaled with hold duration. Time-specific neurons, on the other hand, were responsive only to one time interval. preSMA contained both time-specific and time-graded neurons, whereas SMA primarily showed only time-graded neurons. Additionally, while preSMA neurons demonstrated an instructional response and a movement preparation response, SMA neurons primarily showed only a movement preparation response. This suggests that preSMA neurons are more likely to encode temporal information from instructional cues, and that preSMA and SMA neurons together are involved in action initiation. These findings substantiate those from human fMRI research that medial motor cortical regions play a significant role in interval timing behaviors (Coull et al., 2016).

1.3.7 *Optogenetics*

The ability to transiently activate/inhibit neural subpopulations during behavior has provided a new avenue for investigating the neurobiology of interval timing that was not possible in prior decades. Soares et al. used a discrete trials interval timing task to train mice to discriminate between time intervals that were shorter or longer than a criterion duration by

responding in either a right or left noseport (Soares et al., 2016). By using a number of different durations, the researchers were able to construct psychometric curve of the mice's interval discrimination. Interestingly, optogenetic stimulation of SNc dopamine neurons displaced the psychometric curve rightward such that all but the most extreme intervals were all more likely to elicit a response on the 'short' lever. Optogenetic inhibition of SNc dopamine neurons had a mild, but opposite effect. Additionally, photometric recordings of SNc DA neuron activity revealed a pattern of differential response amplitude depending on interval duration (Soares et al., 2016). These data suggest that SNc dopamine neurons may play an important role in interval timing and discrimination. Whereas optogenetic activation of dopamine neurons in this study led to what could be interpreted as a "slowing down" of time perception, it should be noted that the pharmacological investigations of dopamine and interval timing have often been suggested to show the opposite. As discussed in previous chapters in more detail, global elevations of DA signaling by methamphetamine administration results in a behavioral effect commonly interpreted as a "speeding up" of time perception (Meck, 1996). Pharmacological reduction of DA signaling via haloperidol results in an opposite effect (Buhusi and Meck, 2002; Maricq and Church, 1983). The discrepancy between these optogenetic and pharmacological findings is likely a result of target specificity. Pharmacological administration of dopaminergic drugs affects dopamine signaling throughout the brain, significantly affecting feelings of reward, motivation, arousal, and motor control, all of which may play a role in interval timing behaviors. Although optogenetic manipulations are significantly more specific, SNc DA neurons play a crucial role in non-timing behaviors: reinforcement learning, reward prediction error, and movement regulation, for example (Barter et al., 2015). It is not unreasonable to presume that optogenetic activation of SNc DA neurons might affect these brain functions, and that these changes might in turn

indirectly alter interval timing. In later chapters, I present evidence to suggest that transient, optogenetic alteration of movement alone profoundly affects interval timing behavior.

While the above optogenetic research focused on neural activity within the basal ganglia, Toda et al. used optogenetic manipulations to interrogate the effects of manipulating basal ganglia output signaling on interval timing (Toda et al., 2017). The timing task used by Toda et al. was a peak interval procedure similar to the one discussed previously and discussed in detail in Chapter 2. Mice were trained to lick for a water reward following a fixed interval of time. In probe trials, the water reward was omitted, resulting in a pattern of responding where mice began licking prior to the expected interval and stopped licking sometime after this interval. Across many trials, the histogram of licking across time took the shape of a peak distribution of responding centered roughly around the expected/criterion interval. The authors optogenetically stimulated the terminals of GABAergic neurons projecting from SNr to the superior colliculus. Not only did this stimulation inhibit licking for the duration of stimulation, but it delayed the onset of the start of anticipatory licking for the next trial. Although the authors argue that these results indicate that nigrotectal GABAergic projections are critical for timing behavior, our data presented in Chapter 4 provides an alternative interpretation: it is not the specific pathway that is critical for timing behavior but rather the interrupted ongoing behavior itself. This interpretation hypothesizes, as we found in similar experiments, that if the licking behavior were similarly interrupted via another mechanism, a similar shift in future anticipatory licking would occur.

1.3.7 *Behavioral Strategies*

In the search for neural pathways or processes that govern our sense of time, researchers often encounter a vexing problem: it is difficult to distinguish between a correlate of timing

versus a correlate of a behavior or process that always accompanies such timing (Matell, 2014). This is doubly difficult when looking for timing processes in brain one regions known to be involved in action and motor behavior. To highlight this issue, consider the common interval timing task where mice are trained to time an interval and categorize it as short or long by responding on a left or right lever/noseport. In learning this type of task, it has been observed that mice develop a stereotyped pattern of behavior if left to move freely: at the beginning of the interval, mice first move to the 'short' lever/noseport, and at a middle duration make their way to the 'long' duration lever/noseport (Gouvêa et al., 2014; Howard et al., 2017; Matell, 2014). Indeed, this stereotyped pattern of behavior is so robust that trial-by-trial variation in duration categorization can be predicted by ongoing behavior (Gouvêa et al., 2014). An electrophysiological correlate of interval timing during such a task could therefore be a correlate of the behavior rather than an internal timing process, particularly when looking in regions like the striatum or motor cortices which are known to be involved in action. Matell (2014) recounts an important and amusing discovery in his lab that when trying to eliminate this potential confound (Matell, 2014). To prevent mice from moving about the operant chamber during interval presentation, researchers required mice to hold their snout in a center noseport for the duration of the interval. Although this requirement prevented mice from displaying the previously observed behavior, researchers noticed mice first moving their rump toward one side of the chamber, and then the other side: the behavioral strategy/stereotype was not eliminated, but merely altered. Matell and colleagues therefore hypothesized the so-called 'sphincter problem': any attempt to eliminate a behavioral strategy/stereotype may simply blind the experimenters to the strategy rather than actually eliminate it.

The apparent involvement of motor-related brain regions and the persistence of

stereotypic behaviors in interval timing tasks raises an important question: could animals, and even humans, use something akin to an unconsious behavioral strategy to fundamentally support interval timing? Killeen and Fetterman (1988) proposed one such model of timing that they called the Behavioral Theory of Timing (BeT) (Killeen and Fetterman, 1988). BeT proposes that animals learn to use sequences of behaviors, which they call adjunctive behaviors or behavioral states, to cue the timing of operant responses. This idea comes from the observation that animals engage in repetitive sequences of non-instrumental behaviors during interval timing tasks. BeT proposes that rather than animals actively timing the entire interval, animals chain together these non-instrumental behaviors, the last of which cues an operant response. For example, a rodent may engage in a sequence of behavioral states where, after some cue from the task, they groom, climb on a house-light, walk in a circle around the chamber, then finally make the appropriate response. BeT proposes that 'pulses' from an internal clock guide behavioral state switching rather than act to accumulate passing time as in pacemaker-accumulator type models. A refined version of BeT called Learning to Time (LeT) does not propose the necessity of hypothetical pulses but instead supposes that behavioral states are chained together by associative coupling between one another (Machado, 1997).

Although BeT and LeT provide a framework for how behavioral timing might operate, they appear to necessitate *some* internal process guiding the duration for each behavioral state prior to the operant response (Lejeune et al., 2006). For instance, a period of grooming may be an adjunctive behavior that cues an operant response when completed, but what, in turn, guides the duration of grooming? Another critique of such behavioral theories of timing is that if humans and animals share some fundamental timekeeping mechanism, this would suppose that humans also use learned, adjunctive behaviors to guide timing. It has been argued that this is

unlikely to be true in many cases, given that humans can time appropriately on first exposure to a task without an opportunity for learned, associative coupling of adjunctive behaviors to occur (Lejeune et al., 2006). However, recent work on how young children learn to time has suggested that, indeed, our earliest notions of timing are fundamentally connected to learned actions.

A recent study, substantiating many of the hypotheses of this dissertation, suggests that the selective involvement of motor-related regions during timing tasks is a result of the natural way we learn to time as children (Coull and Droit-volet, 2018). 3 year-old children can learn to perform an action (like squeezing an object) for a specific duration of time, but cannot extend a representation of that learned duration to another action (like pushing a button) (Droit-Volet and Rattat, 1999). Perhaps due to limited cognitive capacity, the learned duration is solely represented by the action. Additionally, duration estimates in these young children are significantly better when the duration is filled with an action rather than with nothing (Droit-Volet, 2008). This research suggests that the developmental origins of our capacity to time intervals may be due to a fundamental coupling of action and time in the brain. If this were true, it would help explain why action-related brain circuits appear to be so intertwined with our ability to time. It could also help explain why, in adulthood, action can certainly improve timing: In rhythmic beat-matching tasks, where subjects listen to a beat and then match it by tapping, it is well established that tapping during the listening phase improves beat matching during the performance phase (Manning and Schutz, 2016). This idea was recently extended to perceptual interval timing, where it was demonstrated that physical beat matching during the learning phase improves the discriminability of that interval on a subsequent perceptual interval timing task (Monier et al., 2019). This intriguing relationship between action and time inspired the motivations and hypotheses of the research presented in this dissertation.

1.4 *Hypothesis*

The primary hypothesis of this dissertation is that the stereotypic behavior widely observed in animal studies of interval timing do indeed contribute to the precision of timekeeping. Numerous lines of evidence suggest a role for motor-related brain structures in interval timing; rather than these structures serving a non-motor function in timing, we propose that these structures may support timekeeping via a mechanism involving learned motor behavior.

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Chapter 2: Single Trials Analysis of Peak Interval Behavior

2.1 *Summary of findings*

Animals have evolved systems for tracking time over an impressively broad range of magnitudes, from circadian timing involved in appetite and sleep/wake behavior to millisecond timing critical for speech and precise motor coordination (Buhusi and Meck, 2005). Timing on the order of seconds-to-minutes – commonly referred to as interval timing – is an equally essential component of everyday behavior. Indeed, *when* to perform an action is often as important as what action to take. Interval timing allows us to perceive and track durations of time, to form temporal associations between events, and to optimize the timing of our decisions. Although numerous brain regions are thought to influence timekeeping, circuits normally associated with action and motor function appear to play a particularly central role (Coull and Droit-volet, 2018; Coull et al., 2011; Gouvea et al., 2015; Merchant and Yarrow, 2016; Rao et al., 2001), notably the dorsal striatum and nigrostriatal dopamine. Yet, the function of these circuits in interval timing has thus far remained unsettled (Coslett et al., 2010; Howard et al., 2017), and it is unclear whether or not they serve a non-motor role in timing.

Researchers have long observed that animals develop a stereotyped pattern of behavior during timekeeping (Gouvêa et al., 2014; Killeen and Fetterman, 1988; Matell, 2014; Skinner, 1948). Historically, this behavior has been viewed as either superstitious or compulsive, with such actions being merely collateral to timekeeping. Here, we take an optogenetic approach to manipulate behavior antecedent to timing decisions. We found that optogenetic stimulation of dorsal striatal D2- and D1-type projection neurons results in identical but very significant changes in both action and timing. We show that these temporal shifts can be replicated by similar optogenetic manipulation of action via a brainstem motor output nucleus, and that

manipulations of nigrostriatal dopamine affect timing only when action is also altered. Together, these results suggest that the link between motor-related brain regions and timing processes exist due to an encoding of time through action rather than due to a non-motor role in time perception.

2.2 *Peak Interval Task Design*

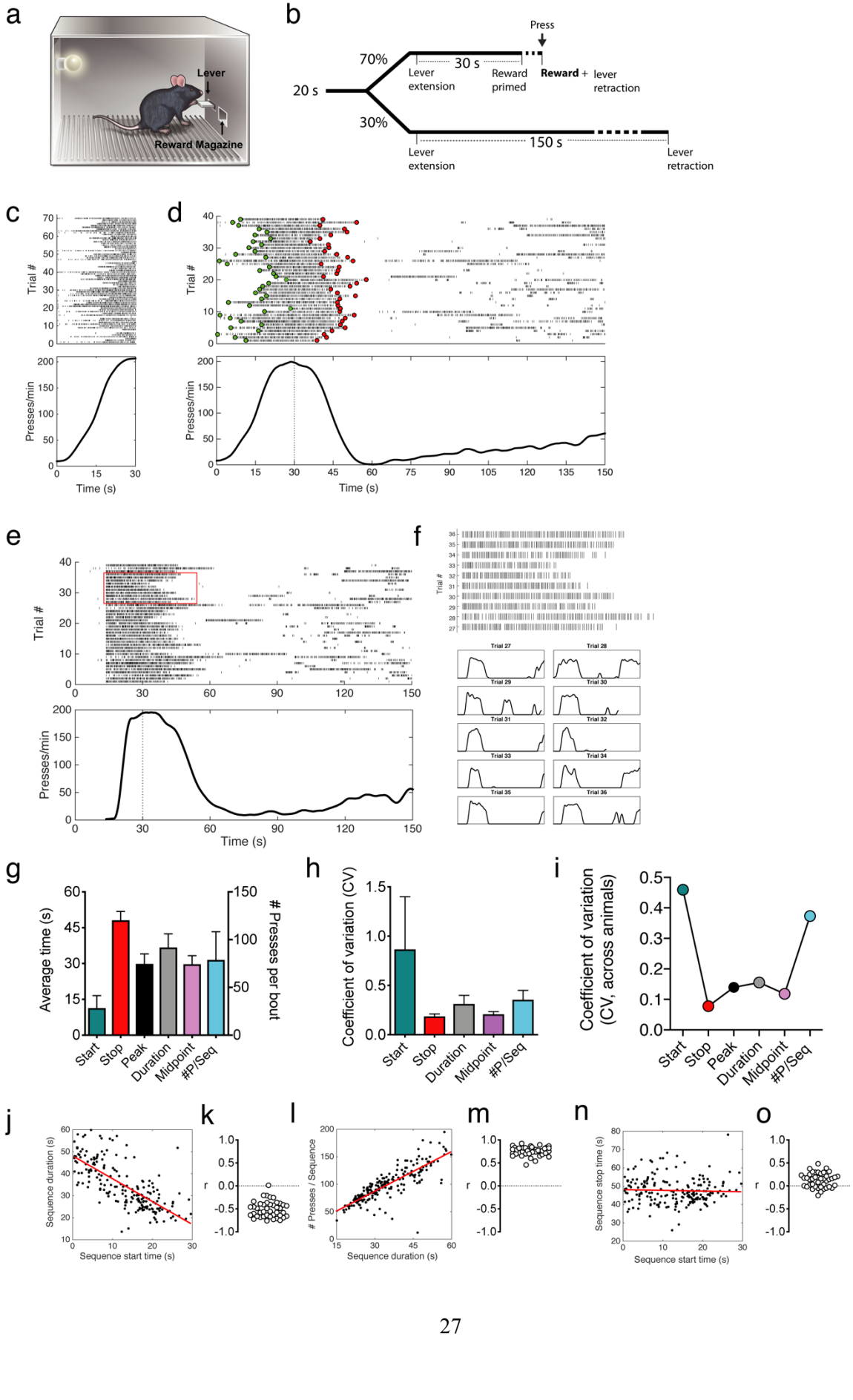
One important behavior that relies on interval timing is foraging, where animals must optimize the duration of food-seeking based upon experience (Bateson, 2003; Brunner et al., 1992). To reproduce this type of emergent timing in the lab, we trained mice on a widely used "temporal foraging" task known as the peak procedure, where animals decide when to start and stop lever pressing for food based on learned temporal task dynamics (Church et al., 1994; Matell et al., 2003; Yin et al., 2016). Mice (n = 42) were trained on the peak procedure operant task to freely forage via lever pressing (Buhusi and Meck, 2005; Gibbon, 1977; Skinner, 1953) (Figure 1A). In an operant box, trials initiated with the extension of a lever. In 70% of trials, the first lever press occurring at least 30 s after trial initiation resulted in the delivery of a food pellet and immediate lever retraction (reward trials). In the other 30% of trials (probe trials), lever pressing never delivered food and the lever remained extended for 150 s before automatically retracting (Figure 1B, see Methods). Reward and probe trials were intermixed randomly so that animals could only discriminate probe from reward trials by tracking time. Consistent with previous observations under similar reinforcement schedules (Buhusi and Meck, 2005; Church et al., 1994; Gibbon, 1977), averaged behavior across reward trials was characterized by an increasing response probability from trial initiation to reward delivery at 30 s (Figure 1C). Averaged behavior across probe trials, on the other hand, displayed the typical bell curve shape, with response probability increasing to a peak at the normal reward delivery time at 30 s, followed by a subsequent decrease in pressing (Figure 1D).

2.3 *Stop time as a trial-by-trial metric for timing*

Traditionally, the 'peak time' of the averaged response function during probe trials has been used to assess animals' timing accuracy (Agostino and Cheng, 2016; Buhusi and Meck, 2005; Maricq and Church, 1983). Although the averaged response function suggests that this peak is formed via an acceleration of pressing as time approaches 30 s and a deceleration afterwards, examining behavior at an individual trial level demonstrates that this is not the case. When we align trial-by-trial behavior to the start time of pressing bouts, the averaged behavior during probe trials takes the shape of a plateau rather than a peak (Figure 1E, F). Indeed, when examining individual trials, press rate does not 'peak' but instead occurs as a single, uninterrupted bout of pressing at a relatively constant rate, starting before and stopping after the criterion duration of 30 s (Figure 1D, F). Variation in these start and stop times creates the epiphenomenon of a peak when averaging across multiple trials. To determine a metric of timing that we could assess on a single-trials level, we performed further analyses on the sequence start time, stop time, duration, and number of presses per sequence for the probe trials across all animals (Figure 1G). We found that both within and across animals, sequence stop time had a significantly smaller coefficient of variation (CV) than sequence start time, sequence duration, or the number of presses per sequence (Figure 1H, I). This suggests that sequence stop time is the most consistent metric of timing on a trial-by-trial basis (Gallistel et al., 2004; Matell and Portugal, 2007). Our analyses revealed a strong negative correlation between sequence duration and sequence start time (Figure 1J, K), as well as a positive correlation between the number of presses per sequence and sequence duration (Figure 1L, M). With a mostly constant press rate, earlier start times thus tend to be accompanied by longer sequences, and later start times by shorter sequences. Indeed, we found sequence stop time to be largely independent of stop time

(Figure 1N, O). So while an animal's internal state and elements of impulsivity may result in the high variation in start time (Matell and Portugal, 2007), these data suggest that a relatively consistent stop time is achieved by modulating the duration of and number of actions within the sequence. On a trial-by-trial level, sequence stop time in probe trials appears to be the best metric of animals' timing, and any changes in timing are therefore best assessed by measuring changes in stop time.

Figure 1 | Single-trials analysis reveals stop time to be a consistent metric of emergent timing. a, Operant box schematic. **b,** Peak-interval operant task design. **c,** Raster (top) and peri-event time histogram (bottom) of presses across rewarded trials of a task session for a representative animal. **d,** Same as **c,** except for probe trials during the task session. Green and red dots indicate sequence start and stop time respectively. **e,** Same data as in **d,** except sequence start times have been aligned to mean start time. Red box indicates trials shown in more detail in **f**. **f,** Raster and histogram for 10 representative start-aligned trials highlighted in **e**. **g,** Mean start, stop, peak, duration, and midpoint times, as well as mean number of presses per sequence for probe trials of all mice (n=42) following training in the task. **h,** Mean within-subject coefficients of variation for the indicated metrics of probe trials. **i,** Across-subject coefficients of variation for the indicated metrics. **j**, Linear regression of sequence start times and duration for all trials of a representative animal. Correlation coefficient: r = 0.734, $P < 0.0001$. **k,** Correlation coefficients of all animals for sequence start time versus duration. **l-m,** same as **j-k**, except for sequence duration versus number of presses/sequence; r = 0.827, $P < 0.0001$. **n-o,** Same as **j-k**, except for sequence start versus stop time; r=0.034, $P = 0.5737$. Data are mean ± s.e.m.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Chapter 3: Optogenetic Manipulation of Striatal Function During Timekeeping

3.1 *Striatal function in timekeeping – action dependence or independence*

Best known for roles in motor learning and action selection, basal ganglia circuitry – and particularly the dorsal striatum – has long been implicated in interval timing in humans and animals (Buhusi and Meck, 2005; Paton and Buonomano, 2018). Electrophysiological recordings have found that the ensemble dynamics of striatal neurons can reflect the encoding of time (Gouvea et al., 2015; Matell et al., 2003; Mello et al., 2015), and various pharmacological manipulations of dorsal striatum appear to affect timing performance (Corte et al., 2019; Meck, 1996). However, it has been widely observed that animals develop a stereotyped pattern of behavior during timekeeping (Gouvêa et al., 2014; Killeen and Fetterman, 1988; Matell, 2014; Skinner, 1948); which in our task manifests as a sequence of lever pressing beginning well before the required operant response (Figure 1D). Whether or not these stereotyped behaviors contribute to interval timing remains unclear. If such actions do serve a function in timekeeping, this raises the possibility that the striatum supports interval timing through its role in action. Alternatively, if emergent stereotyped behaviors do not aid timekeeping, this suggests an action-independent role for striatum in interval timing.

3.2 *Stimulation of D2- and D1-SPNs to alter ongoing pressing behavior*

To test the hypothesis that lever pressing contributes to timekeeping and is not merely an insignificant collateral behavior, we decided to disrupt lever pressing prior to 30 s and assess how stop timing in probe trials was affected. The basal ganglia direct and indirect pathways, originating from striatal D1 or D2-type spiny projection neurons (SPNs) respectively, have been suggested to play distinct roles in controlling actions (Geddes et al., 2018; Kravitz et al., 2010; Tecuapetla et al., 2016). Specifically,

it has been shown that optogenetic stimulation of striatal D2-SPNs inhibits ongoing actions and can cause behavioral switching (Geddes et al., 2018; Kravitz et al., 2010; Tecuapetla et al., 2016).

Taking advantage of this knowledge, we sought to reduce pressing during the waiting period prior to 30 s by optogenetically stimulating D2-SPNs. Optical fibers were bilaterally implanted into the dorsal striatum of mice that genetically expressed ChR2 selectively in D2- or D1-SPNs (Geddes et al., 2018) (Figure 2A-C; see Methods). The functional expression of ChR2 in D2-SPNs was first behaviorally validated in a place preference paradigm. D2-SPN stimulation elicited significant avoidance of the stimulated bisection of the arena (Kravitz et al., 2012), in contrast to the significant preference of mice receiving D1-SPN stimulation or the neutral preference of ChR2-negative mice (2D, E). Following training in the peak-interval task, optogenetic stimulation was delivered in a randomly chosen 50% of trials, both in reward and probe trials such that animals could not discriminate trial type based on stimulation.

3.3 *Behavioral disruption via D2- or D1-SPN stimulation similarly shifts stop time*

We delivered stimulation at different timepoints and for different durations and assessed how these manipulations affected stop timing and the preceding sequence of actions. We first investigated the effects of long (14 s) optogenetic stimulation occurring during either the first or second half of the period preceding 30 s (Figure 2F). Early stimulation of D2-SPNs (0.5 to 14.5 s) dramatically reduced or entirely halted pressing during the stimulation window (Figure 2G), with animals immediately resuming pressing upon laser offset. This stimulation-induced suppression of pressing was not due to the visibility of blue light or disruption of attention, as no behavioral effect was observed during identical photoillumination in ChR2-negative mice (Figure S2). Surprisingly, when we repeated this optogenetic experiment in mice genetically expressing ChR2 selectively in D1-SPNs, we observed nearly identical behavioral effects (Figure 2H,I), despite observing entirely opposite behavioral effects in the place preference paradigm

(Figure 2E). Notably, for stimulation of either striatal population, the suppression of lever pressing from 0.5-14.5 s was accompanied by a delay in stop timing (Figure 2J), suggesting a potential link between stop timing and the preceding behavior.

3.4 *A correlation between behavioral suppression and stop time delay*

To further test this hypothesis, we delivered the same long optogenetic stimulation of D2- or D1-SPNs, this time beginning in second half of the period preceding 30 s. Optogenetic stimulation from 18 s – 32.5 s suppressed lever pressing throughout the stimulation window, and resulted in a delay in stop timing that was significantly longer than the early stimulation despite being of identical duration (Figure 2G-J). Indeed, delayed timing cannot be easily explained by the disruption of attention needed for timekeeping given that identical stimulation durations could result in such greatly different delay magnitudes (Figure 2G, I).

Because animals are more likely to be pressing closer to 30 s, later stimulation resulted in a greater reduction in the number of lever presses than did early stimulation (Figure 2I). When we compared the magnitude of press reduction and the magnitude of the subsequent shift in stop timing, a strong linear correlation emerged (Figure 2K). Greater press reductions resulted in greater stop time shifts, despite stimulation durations remaining the same. This further suggested a possible relationship between stop timing and the preceding sequence of pressing that is independent of any effect of distraction or attention.

3.5 *D1- and D2-SPN Similarly Affect Pressing Behavior and Stop Time Delays*

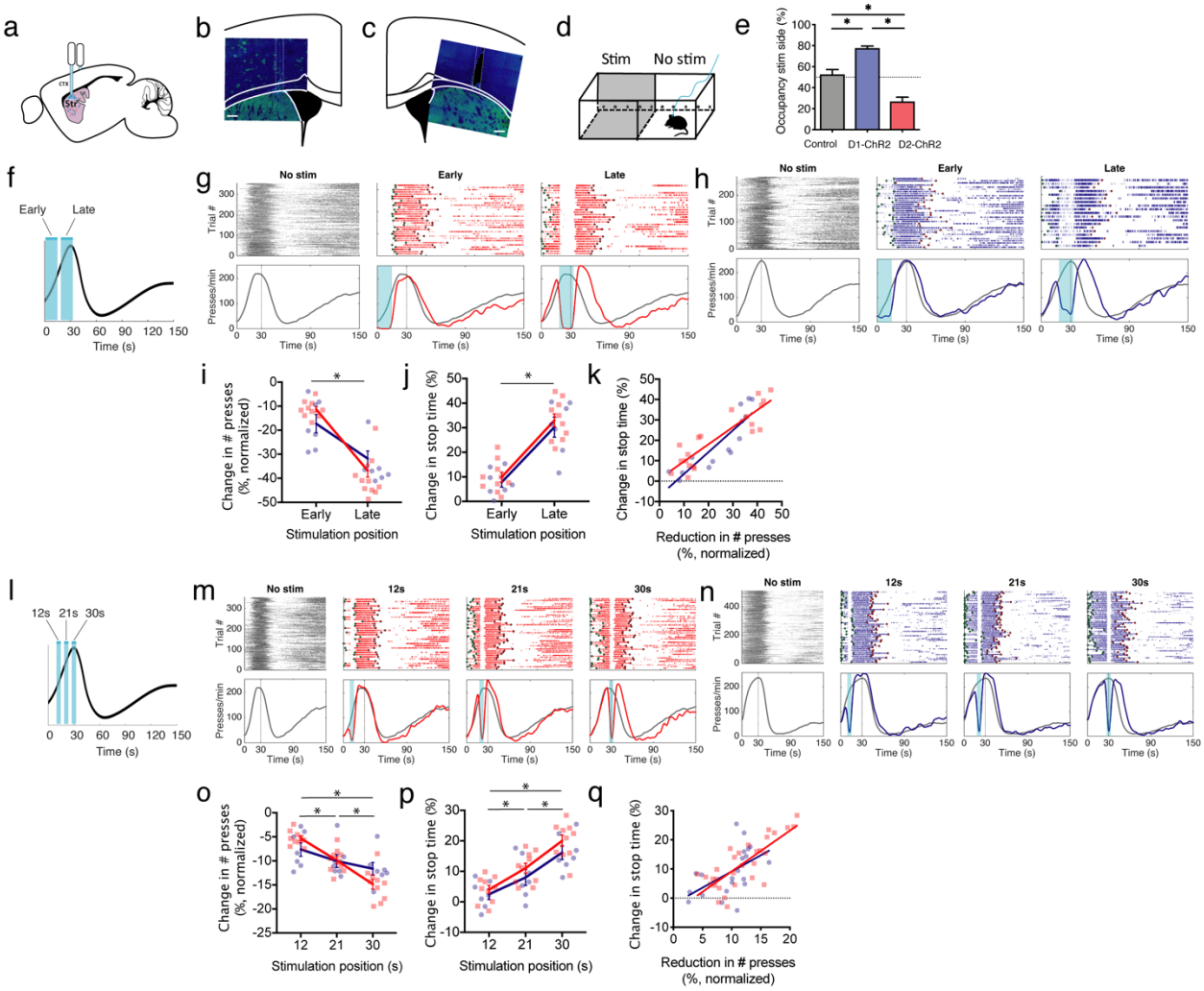
To more precisely investigate the relationship between lever pressing and changes in stop timing, we randomly delivered short (5 s) optogenetic stimulations centered either at 12, 21, or 30 s from

trial initiation (Figure 2L). Stimulation of either D2 or D1-SPNs again suppressed lever pressing during the stimulation window, with pressing again typically resuming immediately after laser offset (Figure 2M, N). Additionally, we again found that, for both populations, stimulation occurring later in the trial resulted in greater number of reduced presses (Figure 2O) and a subsequently larger shift in stop timing (Figure 2P). A strong positive linear correlation between lever press reduction and shift in stop timing was evident for both D2- and D1-stimulation (Figure 2Q).

3.6 *Stimulations of different durations support action-time hypothesis*

When examining the effects of stimulation duration by comparing 14 s and 5 s stimulation windows ending at the same time (either early or late in the trial), it was clear that longer duration stimulation windows reduced more presses and resulted in a greater stop time shifts. (Figure S2). Notably, the delay in stop time in not simply due to a one-to-one making up of the number of presses eliminated by stimulation. For many of the stimulation conditions, the total number of presses per sequence significantly increased (Figure S2). Furthermore, for the same amount of reduced lever presses, optogenetic stimulation occurring later in the trial often resulted in a greater shift in stop time (Figure S2), suggesting that later actions contribute more to the timing of the decision point of when to stop. Together, these data suggest that the highly structured sequence of presses observed in each trial are simply compulsive, collateral actions, but instead may play a role in the timing and the temporal optimization of the animals' decisions.

Figure 2 | Optogenetic stimulation of striatal D2- and D1-SPNs suppresses pressing behavior and delays timing. a, Schematic of implanted optical fibers in dorsal striatum. **b,** Representative eYFP-tagged ChR2 expression and fiber placement in the dorsal striatum of an A2a x Ai32 mouse (D2-ChR2). **c,** Same as b, except for a D1 x Ai32 mouse (D1-ChR2). **d,** Schematic of conditioned place preference arena. **e,** Percent occupancy in the stimulated bisection of place preference arena for either ChR2-negative controls (n=6), D2-ChR2 (n=8), or D1-ChR2 (n=6) groups. **f,** Illustration of the timing of 'early' (0.5 to 14.5 s) or 'late' (18.5 to 32.5 s) optogenetic stimulation following trial initiation. **g,** Rasters and time histograms of lever pressing across trials for a representative D2-ChR2 mouse receiving no stimulation (left), early stimulation (middle), or late stimulation (right). **h,** Same as **g,** but for a representative D1-ChR2 mouse. **i,** Percent change in the number of presses during the stimulation window normalized to mean number of presses per sequence in unstimulated probe trials for D2- (red) and D1-ChR2 (blue) mice during the manipulations shown in **f**. **j,** Percent change in sequence stop time. **k,** Linear regression of the normalized percent reduction in presses during the stimulation window and the percent change in stop times for both early and late stimulations. **l,** Illustration of the timing of optogenetic manipulations examined in **m-q** showing 5s of stimulation centered at 12, 21, and 30 seconds following trial initiation. **m-q,** Same as **g-k**, but using the manipulations shown in **l.** Data are mean ± s.e.m. For other tests of significance for this figure see Methods.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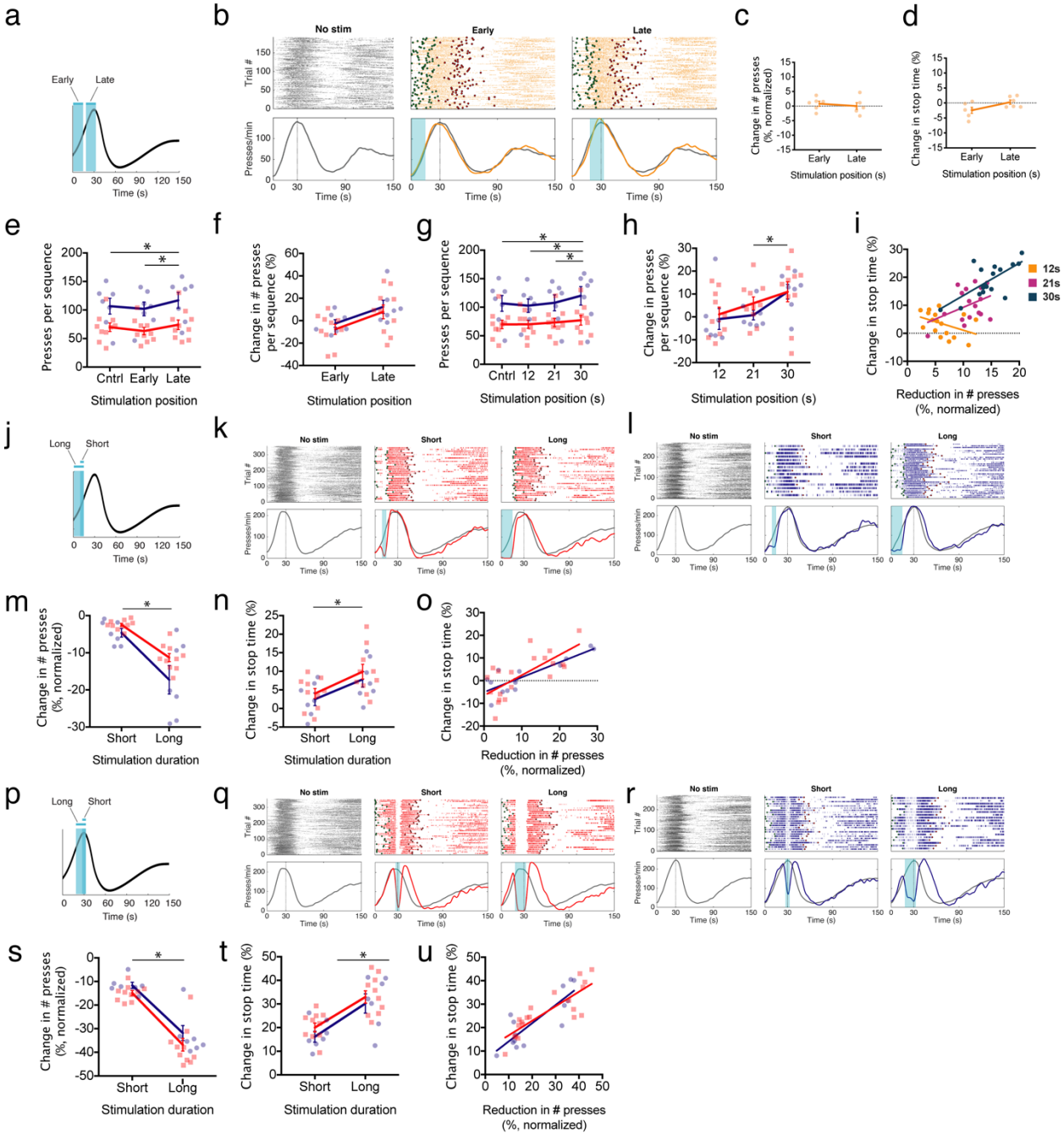
Stimulation position (s)
q
Change in stop time (%)
Reduction in # presses (%, normalized)

Figure S1 | Extended data figure 2. **a,** Illustration of the timing of 'early' (0.5 to 14.5 s) or 'late' (18.5 to 32.5 s) optogenetic photoillumination of the dorsal striatum of ChR2-negative control mice following trial initiation. **b,** Rasters and time histograms of lever pressing across trials for a representative ChR2-negative control mouse receiving no photoillumination (left), early photoillumination (middle), or late photoillumination (right). **c,** Percent change in the number of presses during the photoillumination window normalized to mean number of presses per sequence in unstimulated probe trials for ChR2 negative mice. **d,** Percent change in sequence stop time. **e,** Presses per sequence for D2- (red) and D1-ChR2 (blue) mice receiving no stimulation (Cntrl) or early or late stimulation. **f,** Percent change in the number of presses per sequence for D2- or D1-ChR2 mice receiving stimulation shown in **a. g,** Number of presses per sequence for D2- and D1-ChR2 mice receiving either no stimulation (Cntrl) or 5 s of stimulation centered at either 12, 21, or 30 s from trial initiation. **h,** Same as **f**, but for the 5 s duration stimulations. **i,** Linear regression of normalized reduction in presses during the stimulation window vs. percent change in stop time, broken up by 5 s stimulation position (12, 21, or 30 s) for combined D2- and D1-ChR2 mice. **j,** Illustration of the timing of short (9.5 to 14.5 s, 5 s duration) or long (0.5 to 14.5 s, 14 s duration) early optogenetic stimulation compared in **k-o**. **k,** Rasters and time histograms of lever pressing across trials for a representative D2-ChR2 mouse receiving no stimulation (left), short stimulation (middle), or long stimulation (right) beginning in the first half of the period preceding 30 s. **l,** Same as **k,** but for a representative D1-ChR2 mouse. **m,** Percent change in the number of presses during the stimulation window normalized to mean number of presses per sequence in unstimulated probe trials for D2- (red) and D1-ChR2 (blue) mice during the manipulations shown in **j**. **n,** Percent change in sequence stop time. **o,** Linear regression of the normalized percent reduction in presses during the stimulation window and the percent change in stop times for both short and long stimulations. **p,** Illustration of the timing of short (27.5 to 32.5 s, 5 s duration) or long (18.5 to 32.5 s, 14 s duration) late optogenetic stimulation compared in **q-u**. **q-u,** Same as **k-o**, but using the manipulations shown in **p.** Data are mean ± s.e.m. For other tests of significance for this figure see Methods.



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Chapter 4: Disrupting Motor Behavior in the Brainstem

4.1 *Disrupting action outside of the basal ganglia: the lateral paragigantocellular nucleus*

A wealth of evidence suggests that the striatum is involved in interval timing (Corte et al., 2019; Gouvea et al., 2015; Matell et al., 2003; Meck, 1996). Therefore, one might suggest that our striatal manipulations affect stop timing not by disrupting action, but instead by simply disrupting some non-motor processes in the striatum critical for timing. To address this question, we sought a way to manipulate lever pressing behavior via a motor structure outside of the basal ganglia and its immediate input or output nuclei. One such structure is the lateral paragigantocellular nucleus (LPGi), a motor nucleus in the caudal brainstem which sends direct projections to the spinal cord(Liang et al., 2016). It has recently been reported that LPGi controls locomotion and that optogenetic activation of GABAergic inhibitory neurons in LPGi completely halts movement in mice (Capelli et al., 2017). In vGAT-cre mice, we bilaterally injected AAV-DIO-ChR2 and implanted optical fibers into LPGi (Figure 3A-B, see Methods). We first validated that optogenetic stimulation of LPGi vGAT neurons halted movement in an open field arena (Figure 3C, D).

4.2 *Suppressing action via brainstem delays stop time*

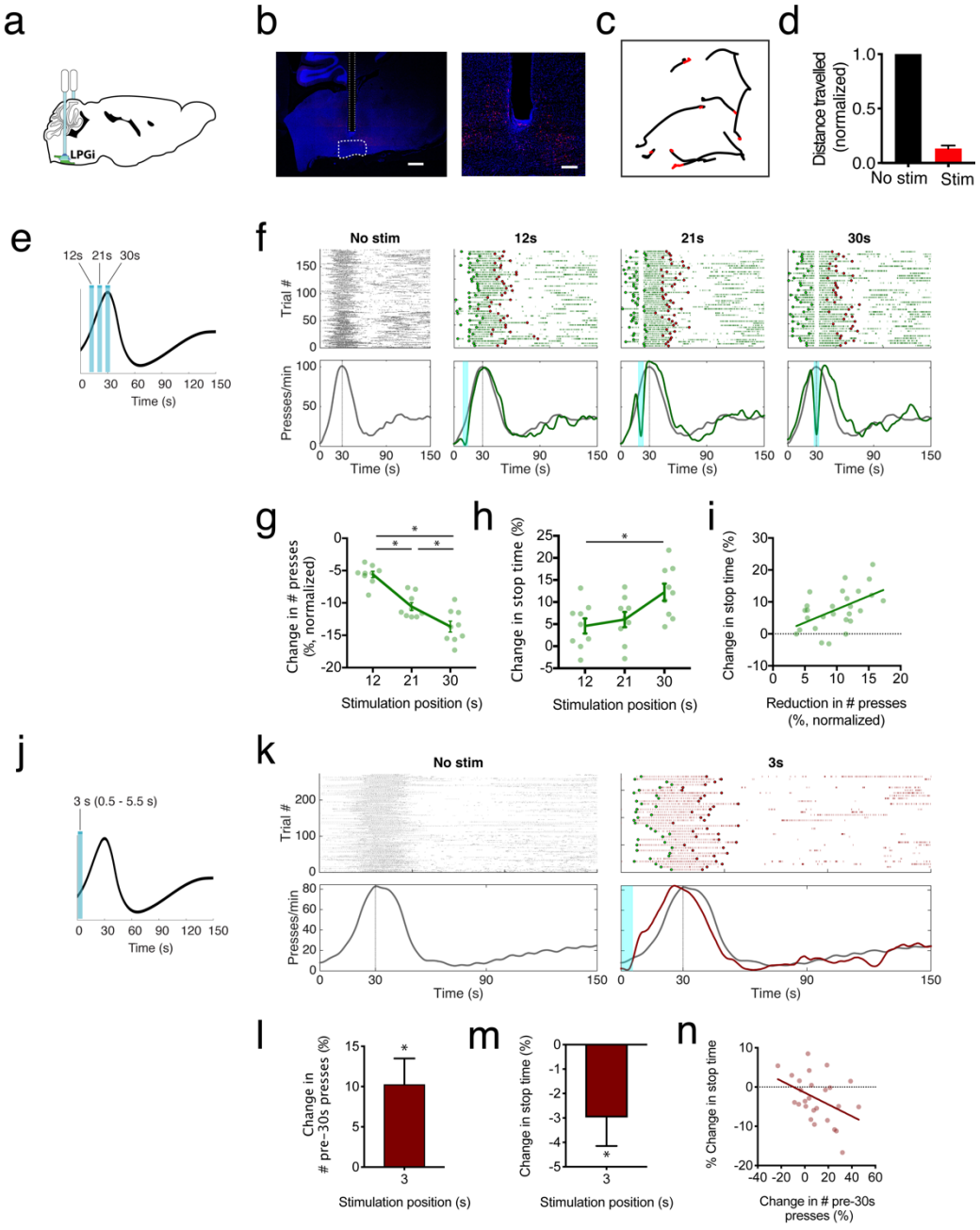
Like in our striatal optogenetic experiments, we randomly delivered short (5 s) optogenetic stimulations centered either at 12, 21, or 30 s from trial initiation (Figure 3C). As we observed with D2- and D1-SPN activation, stimulation of LPGi vGAT neurons strongly suppressed lever pressing during the stimulation window, with later stimulation resulting in greater number of reduced presses than earlier stimulation of the same duration (Figure 3F, G). And again, we observed that this press reduction was followed by a significant delay in stop timing (Figure 3H), with a strong positive correlation between the relative reduction of pressing and the magnitude of stop timing delay (Figure 3I). Additionally, we also

again observed that for a similar reduction of presses, later reduced presses resulted in a greater stop time shift than earlier reduced presses (Figure S3). These results indicate that the shifts in stop time we observed during our striatal D2- and D1-SPN manipulations are not likely due to a direct disruption of temporal processing in dorsal striatum, but instead arise from the disruption of the lever pressing behavior that is often assumed to be merely collateral to timing. Not only do the sequences of lever pressing we observe appear to be necessary for precise timekeeping, but there also appears to be a non-linear relationship between lever pressing and timekeeping such that later actions contribute more to timing.

4.3 *Increasing cumulative action accelerates stop time*

Given our observation that reducing lever presses delays stop time, one might predict that experimentally increasing lever pressing would result in earlier stop timing. To test this, we employed a stimulation protocol that we found to reliably increase cumulative lever pressing prior to 30 s in all previously examined groups of mice. A short (5 s) optogenetic stimulation was delivered 0.5 s after trial initiation (Figure 3J). We observed once again that lever pressing was reduced during the stimulation window (Figure 3K) – however, after stimulation offset, we observed a consistent increase in the number of lever presses prior to the normal reward delivery time of 30 s (Figure 3L). This increase in pressing was followed by a significant decrease in stop time (Figure 3M), again demonstrating a correlation between lever press changes and stop time shifts (Figure 3N). These results suggest that stop timing is significantly influenced by preceding lever pressing, and that bidirectional facilitation or inhibition of these actions can bidirectionally regulate stop timing.

Figure 3 | Bidirectional modulation of action is sufficient at bidirectionally shifting timing. **a,** Illustration of optical fibers implanted bilaterally above the lateral paragigantocellular nucleus (LPGi). **b,** Histology showing fiber tract above LPGi and expression of virally delivered mCherry-tagged ChR2 in a representative vGAT-cre mouse. **c,** Movement tracks of a representative mouse in an open field arena 5 s prior (black) and 5 s during (red) LPGi-vGAT stimulation. **d,** Normalized distance travelled 5 s prior to and 5 s during LPGi-vGAT stimulation (n=3). **e,** Illustration of the timing of the optogenetic manipulations examined in **f-i**, showing 5 s of stimulation centered at 12, 21, and 30 s following trial initiation. **f,** Rasters and time histograms of lever pressing across trials for a representative mouse receiving no stimulation (leftmost panel) or 5 s of LPGi-vGAT stimulation centered at 12, 21, and 30 s respectively. **g,** Percent change in the number of presses during the stimulation window normalized to baseline number of presses per sequence. **h,** Percent change in stop time across stimulation positions. **i,** Linear regression of the normalized percent reduction in presses during stimulation and the percent change in stop times. **j,** Illustration of the timing of early-in-trial optogenetic manipulation, showing 5 s of constant photoillumination centered at 3 seconds (0.5 s to 5.5 s) following trial initiation. Data from striatal D1-SPN and D2-SPN and LPGi-vGAT stimulation was pooled due to no significant difference in stop time shift for all stimulation positions (n=23). **k,** Raster and time histogram of lever pressing across trials for a representative mouse receiving no stimulation (leftmost panel) or the stimulation illustrated in **j. l,** Percent change in the cumulative number of pre-30 s lever presses in stimulation trials. **m,** Percent change in stop time. **n,** Linear regression of the percent change in culmulative pre-30 s presses vs. percent change in stop time.



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Chapter 5: Stimulating SNc Dopamine Neurons during Timekeeping

5.1 *Dopamine in timing and motor control – a possible intersection*

Dopamine has been implicated in interval timing by both clinical observations of Parkinson's patients(Honma et al., 2016; Malapani et al., 1998, 2002; Pastor et al., 1992) and numerous animal studies using pharmacologicald (Cheung et al., 2006; Lake and Meck, 2013; Maricq and Church, 1983), genetic (Balci et al., 2010; Drew et al., 2007), and optogenetic manipulations (Soares et al., 2016). However, dopamine is also known to play a critical role in action selection and voluntary movement control (Barter et al., 2015; Howard et al., 2017; Jin and Costa, 2010; Da Silva et al., 2018). Our results so far have indicated that action can be an essential component of timekeeping, and that manipulating such actions can correspondingly alter timing. It thus remains unclear whether dopamine has a direct, non-motor role in controlling time perception (Soares et al., 2016), or whether dopaminergic manipulations affect interval timing by altering timekeeping actions. If the latter case were true, one would expect that the manipulation of dopamine signaling would alter interval timing if, and only if, timekeeping actions were also altered.

5.2 *Validation of optogenetic stimulation of dopamine: voltammetry and place preference*

To test this hypothesis, we decided to optogenetically stimulate dopaminergic neurons in bilateral substantia nigra pars compacta (SNc) (Howard et al., 2017) in mice trained in the peak-interval task (Figure 4A-B, see Methods). Two stimulation conditions were used for evoking different degrees of dopamine release: a low intensity protocol (20 Hz, 5 mW) and a high intensity protocol (50 Hz, 15 mW) d(Howard et al., 2017). The functional release of dopamine by optogenetic stimulation was first validated in the conditioned place preference (CPP) test (Figure 4C). Stimulation using the high intensity protocol in one bisection of the area resulted in a strong place preference that was strongly and consistently

reversed the following day using the low intensity protocol (Figure 4D). This indicated that that both stimulation protocols evoked dopamine release and were sufficient at inducing place preference. To further characterize the dopamine release elicited by the two optogenetic stimulation protocols, a carbon fiber microelectrode was implanted into the dorsal striatum to record subsecond dopamine release using fast-scan cyclic voltammetry(Howard et al., 2017) (see Methods). Optogenetic stimulation using the high intensity protocol evoked significant dopamine release while the mouse performed in the peak-interval task (Figure 4E, F).

5.3 *High but not low intensity SNc stimulation suppresses action and delays stop time*

When this stimulation protocol was delivered at 12 s, 21 s, and 30 s, lever pressing was significantly depressed during the stimulation windows (Figure 4G, H). Notably, this reduction is pressing was accompanied by significant delays in stop time that linearly increased according to when in the trial stimulation took place (Figure 4I). We once again observed a positive correlation between the magnitude of press reduction and the magnitude of stop time delay (Figure 4J).

Optogenetic stimulation under the low intensity protocol also significantly evoked dopamine release in dorsal striatum as mice performed in the task (Figure 4K, L). However, this lower intensity stimulation protocol failed to suppress lever pressing (Figure 4M, N). Notably, there were no changes in stop time at any of the three stimulated time points (Figure 4M, O), despite the fact that this lower intensity stimulation did elicit significant dopamine release and was able to strongly reverse place preference induced by the higher stimulation protocol. Thus, we found that our dopaminergic manipulations affected timekeeping only insofar as the actions during the timing period are also affected, suggesting a crucial role for these actions in interval timing in our task.

5.4 *A motor mechanism for timekeeping*

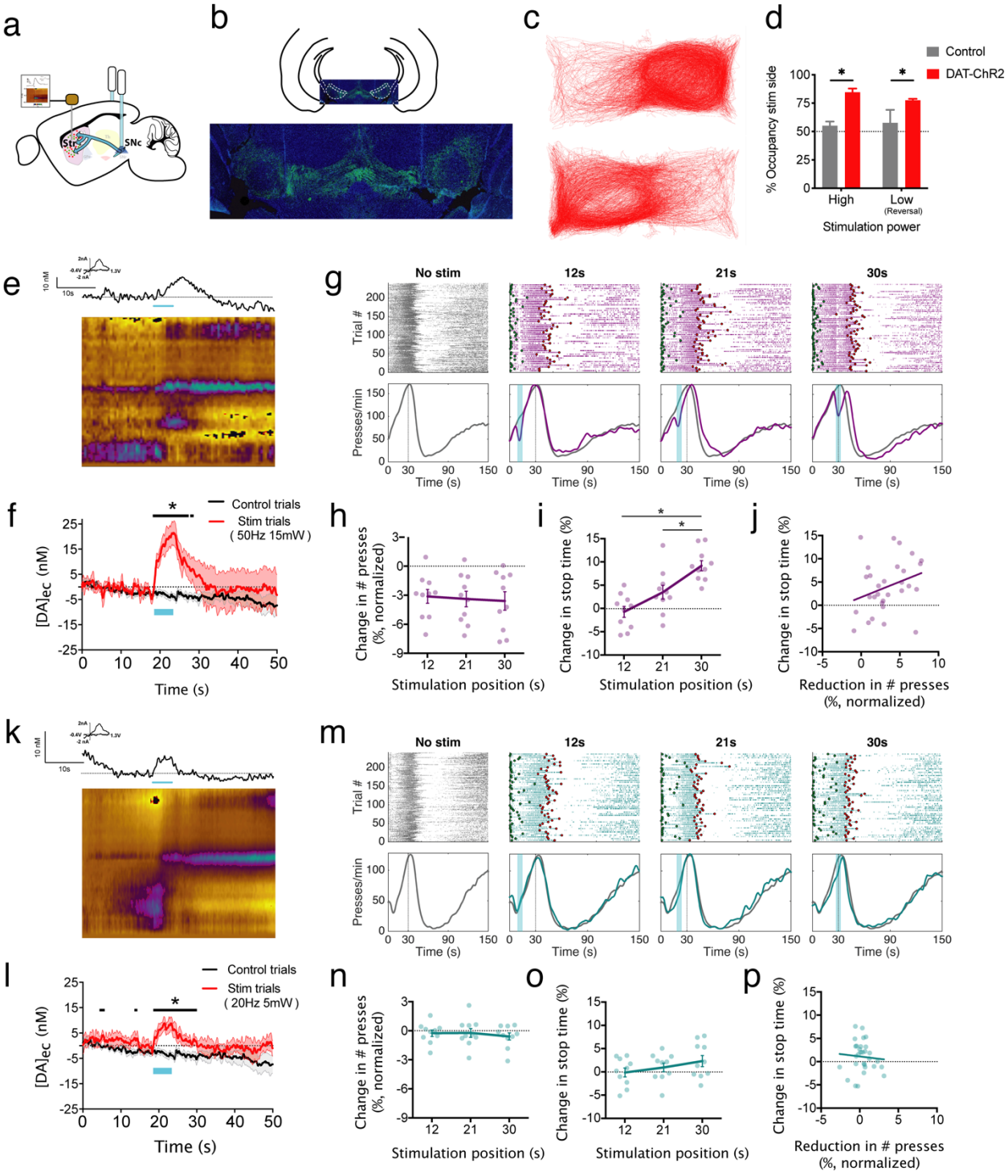
The emergence of stereotyped patterns of behavior is a common phenomenon in interval timing tasks (Killeen and Fetterman, 1988; Matell, 2014). Although behavioral theories of timing have been previously proposed (Killeen and Fetterman, 1988; Machado, 1997), experimental evidence for behavioral timing has been sparse. Here, we show that interval timing can be significantly distorted by a transient disruption to behavior antecedent to a temporal judgment, and that the degree of temporal distortion is strongly correlated with the magnitude of behavioral manipulation. The dorsal striatum has long been implicated in interval timing, but no study to date has assessed the timing effects of selective activation of either of the two canonical basal ganglia pathways, the D1 and D2-SPN mediated direct and indirect pathways. We demonstrate that activation of either pathway, surprisingly, results in a similar effect on both ongoing behavior and interval timing. To assess whether timing effects we observed were a result of a manipulation of action or a result of disrupting a non-motor timing function of dorsal striatum, we replicated the observed behavioral phenotype via activation of an inhibitory motor output nucleus in the caudal brainstem. Indeed, manipulation of motor behavior via the brainstem was sufficient at achieving nearly identical distortions in interval timing as we observed with D1- or D2-SPN activation, suggesting a potentially important role for action in timing. Prior pharmacological, genetic, and optogenetic studies have indicated a role dopamine in interval timing. However, recent studies have also demonstrated a role for dopamine in movement control (Barter et al., 2015; Da Silva et al., 2018). By optogenetically evoking dopamine to different degrees during our task, we demonstrate that interval timing is distorted by dopaminergic activation only when such activation is sufficiently strong so as to affect motor behavior.

Interval timing is well known to be affected by attentional, emotional, and sensory contexts (Coull et al., 2004; Droit-volet, 2018). One might argue that the temporal distortions we see are simply a

result of disrupted attention. However, a number of observations suggest otherwise – first during our optogenetic manipulations of ongoing behavior, suppression of pressing is not always entirely halted. In these instances, as can be observed in the raster plots of behavior for striatal D1-SPN (Figure 2H) and high power SNc DAT stimulation (Figure 4G), animals' press rate is depressed, but they still remain engaged with the lever. This suggests that their attention has not been disrupted to the point of task disengagement. Moreover, for virtually all mice used in our experiments, lever pressing following behavioral inhibition resumed nearly immediately after laser offset. If stimulation significantly disrupted animals' attention to the task, one would arguably expect the latency of resuming pressing to be much longer. Additionally, in Figure 3, we demonstrate that by facilitating early engagement with the lever by inhibiting action just after trial initiation, we are able to observe a significant acceleration of stop time rather than a delay. If the stop time delays we observe after later press suppression are a result of disrupted attention, one would also expect a stop time delay with very early stimulation. Instead, we see that when pressing is increased prior to 30 s, rather than decreased, stop time occurs earlier.

Our data provides evidence that behavior once thought to be collateral to timing indeed may serve a timekeeping function. Moreover, by demonstrating a relationship between motor behavior and timekeeping, our data provides a key insight into why motor related brain circuits throughout the brain have been found to play a significant role in interval timing.

Figure 4 | Stimulation of dopamine neurons in SNc delays timing only when action is transiently disrupted. a, Schematic showing implanted optical fibers above the substantia nigra pars compacta, and carbon fiber voltammetry electrodes implanted in dorsal striatum. **b,** Histology showing YFP-tagged ChR2 expression in SNc and optical fiber tracts above. **c,** Movement tracks of a representative DAT-ChR2 mouse receiving 50 Hz 15mW SNc stimulation ("high power") on one half of a place preference arena (top) and 20 Hz 5 mW stimulation ("low power") the next day on the opposite half of the arena (bottom). **d,** Percent occupancy of the stimulated bisection of the place preference arena for ChR2-negative controls (n=5) or DAT-ChR2 mice receiving SNc stimulation as described in **c**. **e,** Representative real-time changes in dopamine concentration in dorsal striatum during 5 seconds of SNc stimulation at 50 Hz 15 mW while the animal is performing the task. Black line shows dopamine concentration, and psuedocolor plot shows current recorded at each time point in the voltage sweep across time. **f,** Mean real-time changes in calculated dopamine concentration in dorsal striatum during 50 Hz 15 mW stimulated trials (red) or non-stimulated trials (black). **g,** Rasters and time histograms of lever pressing across time for a representative mouse receiving no stimulation (left) or 5 s of 50 Hz 15 mW stimulation centered at 12, 21, or 30 s respectively. **h,** Percent change in the number of presses during stimulation relative to baseline number of presses per sequence for mice receiving 50 Hz 15 mW stimulation. **i,** Percent change in sequence stop time following 50 Hz 15 mW stimulation. **j,** Linear regression of the normalized percent reduction in presses and the percent change in stop times for all 50 Hz 15 mW stimulations. **k-p,** Same as **e-j,** except for 20 Hz 5 mW stimulation.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Appendix: Methods

A.1 *Subject Details*

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Salk Institute and were in accordance with the National Institute of Health's Guide for the Care and use of Laboratory Animals. Male and female mice of at least two months of age and with a C57BL/6J background (Jackson Laboratory) were housed on a 12 hr light/dark cycle with lights on at 6:00 am. D1-Cre (GENSAT: EY217), A2a-Cre (GENSAT: KG126), vGAT-Cre (GENSAT: ????), and DAT-Cre mice obtained from MMRRC were crossed to Ai32 (Jackson Laboratory: 024109) mice that express channelrhodopsin-2/EYFP upon exposure to cre (Jin et al. 2014; Tecuapetla et al. 2016; Madisen et al. 2012).

A.2 *Behavioral Setup and Training*

As previously described, training took place in a standard operant chamber (21.6 L, 317.8 W, 312.7 H cm; Med Associates, St. Albans, VT) within a sound attenuating box (Jin et al. 2010; Jin et al. 2014; Howard et al. 2016). The operant chamber contained a single retractable lever to the left of a central food magazine, and a house light (3V, 24V) was positioned centrally in the back of the chamber. Upon reward, a single 20mg food pellet (Bio-Serv, NJ) was dispensed into the food magazine. An infrared sensor within the magazine detected head entries. Operant boxes were automatically controlled by Med Associates behavioral software (MED-PC IV) using custom written behavioral programs. Timestamps were recorded at 10ms resolution for all lever presses and head entries.

Mice were food restricted for at least 24 hrs prior to training and were maintained at 80-85% of their free-feeding body weight throughout training. Mice were first exposed to continuous reinforcement (CRF) training where one reward was given for every lever press, for a

maximum of 15 rewards per 60 minute session. Mice remained on this schedule until all 15 rewards were received in 15 minutes or under (usually 3 days).

Following CRF training, mice were trained on a modified version of the classical peak-interval task (citation). Trials consisted of either rewarded trials (70% probability) or unreinforced 'probe' trials (30% probability). Both probe and rewarded trials began with the extension of the lever. In rewarded trials, the first lever press occurring at least 30 s following lever extension resulted in pellet delivery and immediate lever retraction, marking the end of the trial. Lever pressing prior to the 30 s criterion was recorded but had no effect. In probe trials, no pellets were ever given, and the lever remained out and inactive for 150 s before automatically retracting and marking the end of the trial. Inter-trial-intervals after both reward and probe trials were fixed at 20 s. Sessions continued until mice received 70 pellets or until the session expired at 180 minutes. Mice were trained for at least 28 days prior to optogenetic experimentation.

A.3 *Surgical Procedures*

For optogenetic experiments, fiber optic implants were constructed and implanted as previously described (Howard et al., 2016; Tecuapetla et al. 2016). Briefly, mice were anesthetized with isoflurane (4% induction; 1%-2% sustained) and positioned on a stereotactic frame. Optical fibers (200um) affixed to ceramic ferrules were chronically implanted into the brain and were affixed to the skull using Opti-bond self-etching adhesive (Kerr Dental) and Tetric Evoflow light-curable dental cement (Ivoclar Vivadent). Optical fibers were bilaterally implanted relative to Bregma at: Striatum (0.5 mm anterior, 2.0 mm lateral, and 2.2 mm ventral); Substantia nigra pars compacta, (3.2 mm posterior, 1.2 mm lateral, 3.9 mm ventral). Relative to lambda: Lateral gigantocellular reticular nucleus (2.0 mm posterior, 1.0 mm lateral, 5.2 mm ventral)

For simultaneous optogenetic and fast-scan cyclic voltammetry experiments, custom carbon fiber voltammetry electrodes were chronically and bilaterally implanted into dorsal striatum as previously described (Howard et al., 2016; relative to Bregma at 0.5 mm anterior, 2.0 mm lateral, and 2.2 mm ventral). Chloridized silver reference electrodes were implanted ipsilateral to their respective recording electrode. Reference and recording electrodes were attached to connector extending out of the dental cement that allowed a voltammetry head stage to be connected to the mouse during recording sessions. Optical fibers were implanted above SNc (relative to Bregma at 3.1-3.4 mm posterior, 1.0-1.5 mm lateral, 3.6 mm ventral) and were incrementally lowered until a robust evoked dopamine response was detected in dorsal striatum. Optical fibers and voltammetry electrodes were then affixed to the skull with dental cement. Following all surgeries, mice were administered buprenorphine (1mg/kg) and were allowed at least one week to fully recover before food restriction and the initiation of behavioral training.

A.4 *Optogenetic Experiments*

Optogenetic stimulation in the peak-interval task began after least 28 days of training, which included at least one week of training with fiber optic cables attached. Stimulation data was collected across multiple days to gather enough data for analysis. On stimulation days, stimulation occurred on 50% of both rewarded and probe trials. Stimulation was always bilateral, and 473 nm light was used in all experiments. A pulse width of 10ms was used for all stimulation patterns at frequency unless otherwise specified.

For *D1-Ai32* and *A2a-Ai32* mice receiving stimulation in striatum, stimulation consisted of constant illumination at 1mW (LaserGlow Technologies). The particular stimulation window was randomly chosen on a trial-by-trial basis within sessions. 5 s stimulation windows were centered at either 3, 12, 21, or 30 s from trial initiation (occurring, respectively, from 0.5 to 5.5 s,

9.5 to 14.5 s, 18.5 to 23.5 s, or 27.5 to 32.5 s). 14 s stimulation windows occurred from either 0.5 to 14.5 s or 14.5 to 32.5 s, matching stimulation endpoints from the 5 s stimulation windows. Stimulation sessions continued until mice received 90 food pellets or until the session expired at 240 minutes. For *LPGi-vGAT* mice receiving stimulation in LPGi, stimulation consisted of constant illumination at 5 mW. Mice in these experiments received 5 s stimulation windows centered either at 12, 21, or 30 s. For *DAT-Ai32* mice receiving stimulation in SNc, stimulation consisted of either 20hz illumination at 5mw or 50hz illumination at 15mw. Mice in these experiments received stimulation during all windows as D1- and A2a-Ai32 mice.

A.5 *Fast-Scan Cyclic Voltammetry (FSCV)*

Dopamine was recorded using FSCV as previously described, using potentiostats and head-stages custom fabricated by the Department of Psychiatry & Behavioral Sciences at the University of Washington, and using Tarheel software (ESA Biosciences) (Clark et al., 2010). Prior to each recording session, the voltammetric head stage was attached to the mouse in its homecage, and a triangular waveform (-0.4 to 1.3 to -0.4 V at 400 V/s) was applied to the tip of the carbon fiber electrode for one hour at 60hz, followed by 30 minutes at 10hz or until a stable background was present. Only electrodes with a background of 200nA or greater and a signal:noise ratio above 250:1 were used. Following this cycling period, mice were transferred to the operant box and the 10 Hz triangular waveform was applied throughout the behavioral session. For each recording, principal component analysis (PCA) (Keithley et al., 2009) was used to isolate changes in current caused by changing dopamine concentrations from changes in current due to pH or electrode drift. Changes in background current were collected on each recording day for each electrode used, providing a unique PCA training set for electrode drift for each recording. PCA training sets for dopamine and pH changes were constructed from in vitro

recordings in a flow cell apparatus using glass electrodes. Recorded current was converted to concentration using background current and an equation derived from in-vitro recordings using glass-sealed carbon fiber electrodes. Analysis of recorded voltammetry data was performed using HDCV software provided by the Department of Chemistry at the University of North Carolina at Chapel Hill (Bucher and Wightman, 2015; Howard et al., 2013).

A.6 *Trial-by-Trial Analysis*

Start and stop times of individual pressing bouts within trials were identified automatically during data analysis. Start time was marked as the first press of a trial that was followed by at least three consecutive presses separated by inter-press-intervals less than 2 s. Trials with start times greater than 30 s were excluded from further analysis. Stop time was marked as the first press that was followed by at least 4 s of no pressing that also met the following criteria: eligible stop times were restricted to those presses occurring after the median press time for a given trial, as well as those presses occurring before 80 s from trial initiation. These restrictions were put in place to avoid marking stop times due to early sample pressing, and to reduce noise created by the highly variable bout-independent pressing that occurred near the end of trials. Trials where no stop time was found were excluded from further analysis. Additionally, any trial with less than 10 presses total was considered an 'unengaged' trial and was excluded from analysis.

A.7 *Statistical Analyses*

Statistical analyses and details for specific experiments were performed as described below.

Figure 2e: Percent occupancy in the stimulated bisection of place preference arena for

either ChR2-negative controls (n=6), D2-ChR2 (n=8), or D1-ChR2 (n=6) groups. One-way ANOVA, F (2, 17) = 44.42, $P < 0.0001$. Tukey's multiple comparisons test, Control vs. D1-ChR2, $P < 0.0012$, Control vs. D2-ChR2, $P < 0.0005$, D2-ChR2 vs. D1-ChR2, $P < 0.0001$.

Figure 2i: Percent change in the number of presses during the stimulation window normalized to baseline number of presses per sequence for A2a- (red) and D1 x Ai32 (blue) mice during early and late stimulation. Two-way repeated measures ANOVA, Main effect of stimulation position, F (2, 30) = 140.7, P<0.0001. Tukey's multiple comparisons post hoc tests. All comparisons significant, $P<0.0001$.

Figure 2j: Percent change in sequence stop time for A2a and D1 x Ai32 mice. Two-way repeated measures ANOVA, Main effect of stim position, F (2, 30) = 134.1 P<0.0001.

Figure 2k: Linear regression of the normalized percent reduction in presses during the stimulation window and the percent change in stop times for both early and late stimulations. D1 x Ai32: correlation coefficient r = 0.8783, P<0.0001 ; A2a x Ai32: correlation coefficient r = 0.8875, $P<0.0001$.

Figure 2o: Percent change in the number of presses during stimulation normalized to baseline number of presses per sequence for A2a- and D1 x Ai32 mice receiving 5 seconds of stimulation centered at 12, 21, and 30 seconds from lever extension. Two-way repeated measures ANOVA, main effect of stimulation position, F (4, 60) = 105.6, $P<0.0001$. Tukey's multiple comparisons post hoc tests: all comparisons significant.

Figure 2p: Percent change in stop time across stimulation positions. Two-way repeated measures ANOVA, main effect of stimulation position, F (4, 60) = 60.02, $P<0.0001$. Tukey's multiple comparisons post hoc test: all comparisons significant.

Figure 2q: Linear regression of the normalized percent reduction in presses and the

percent change in stop times for all manipulations shown in **l**. D1 x Ai32: correlation coefficient r = 0.5264, P=0.0142 ; A2a x Ai32: correlation coefficient r = 0.7825, $P<0.0001$.

Figure S2c: **,** Percent change in the number of presses during the photoillumination window normalized to mean number of presses per sequence in unstimulated probe trials for ChR2 negative mice. One-way ANOVA, no significance.

Figure S2d: Percent change in sequence stop time for ChR2-negative mice. One-way ANOVA, no significance.

Figure S2e: Presses per sequence for D2- (red) and D1-ChR2 (blue) mice receiving no stimulation (Cntrl) or early or late stimulation. Two-way repeated measures ANOVA. Main effect of genotype ($F (1, 15) = 8.244$, $P = 0.0117$) and stimulation position ($F (2, 30) = 12.07$, $P=0.0001$). Tukey's multiple comparisons post hoc tests. Cntrl vs. Late, $P = 0.0284$. Early vs Late, $P<0.0001$.

Figure S2f: Percent change in the number of presses per sequence for D2- or D1-ChR2 mice. Two-way repeated measures ANOVA, main effect of stimulation position $F (1,15) = 20.76$, $P=0.004$.

Figure S2g: Number of presses per sequence for D2- and D1-ChR2 mice receiving either no stimulation (Cntrl) or 5 s of stimulation centered at either 12, 21, or 30 s from trial initiation. Two-way repeated measures ANOVA. Main effect of genotype ($F (1, 15) = 6.457$, $P = 0.0226$) and stimulation position ($F (3, 45) = 10.74$, $P<0.0001$). Tukey's multiple comparisons post hoc tests. Cntrl vs. 30, $P = 0.0003$. 12 vs 30, $P<0.0001$, 21 vs 30 $P = 0.0056$.

Figure S2h: Same as Fig. S2**f**, but for the 5 s duration stimulations. Two-way repeated measures ANOVA. Main effect of stimulation position $F (2,30) = 5.527$, $P = 0.0090$. Tukey's repeated measures post hoc tests. 12 vs 30 $P = 0.0080$.

Figure S2i: Pearson correlations of normalized reduction in presses during the stimulation window vs. percent change in stop time, broken up by 5 s stimulation position (12, 21, or 30 s) for combined D2- and D1-ChR2 mice. 12 s, r = -3985, ns, $P = 0.1131$. 21 s, r = 0.3986, ns, $P = 0.1131$, 30 s, r = 0.6933, $P = 0.0020$ (all two-tailed).

Figure S2m: Percent change in the number of presses during the stimulation window normalized to baseline number of presses per sequence for D2- and D1-ChR2 mice during short and long early stimulation terminating at the same time. Two-way repeated measures ANOVA, main effect of stimulation position, $F(2, 30) = 71.61$, $P<0.0001$.

Figure S2n: Percent change in sequence stop time during the manipulations shown in **j**. Two-way repeated measures ANOVA, main effect of stimulation position, $F(2, 30) = 134.1$ $P<0.0001$.

Figure S2o: Linear regression of the normalized percent reduction in presses during stimulation and the percent change in stop times for all manipulations shown in **j**. D1-ChR2: correlation coefficient r = 0.8572, $P<0.0001$; D2-ChR2: correlation coefficient r = 0.7109, $P = 0.0004$.

Figure S2s: Percent change in the number of presses during stimulation normalized to baseline number of presses per sequence for D2- and D1-ChR2 during short and long late stimulation terminating at the same time, as shown in **p**. Two-way repeated measures ANOVA, main effect of stimulation position, $F(2, 30) = 258.1$, $P<0.0001$.

Figure S2t: Percent change in the number of presses per sequence across short and long late stimulation. Two-way repeated measures ANOVA, main effect of stimulation position, $F(2, 30) = 150.8$, $P<0.0001$.

Figure S2u: Linear regression of the normalized percent reduction in presses and the

percent change in stop times for all manipulations shown in S2**p**. D1-ChR2: correlation coefficient r = 0.8446, P=0.0001 ; D2-ChR2: correlation coefficient r = 0.8337, P<0.0001 .

Figure 3g: Percent change in the number of presses during the stimulation window normalized to baseline number of presses per sequence. One-way ANOVA, main effect of stimulation position, F (1.816, 9.08) = 112.2, P<0.0001. Tukey's multiple comparisons post hoc tests: all comparisons significant.

Figure 3h: Percent change in stop time across stimulation positions. Two-way repeated measures ANOVA, main effect of stimulation position,. Tukey's multiple comparisons post hoc test: 12 s vs 30 s, *P*<0.0001.

Figure 3i: Linear regression of the normalized percent reduction in presses during stimulation and the percent change in stop times. Correlation coefficient r = 0.5219, *P* = 0.0052.

Figure 3l: Percent change in the cumulative number of pre-30 s lever presses in stimulation trials. One-sample t test (theoretical mean 0.00), *P* = 0.0037 (two-tailed).

Figure 3m: Percent change in stop time. One sample t test (theoretical mean 0.00), *P* = 0.0169 (two-tailed)

Figure 3n: Linear regression of the percent change in cumulative pre-30 s presses vs. percent change in stop time. Correlation coefficient r = -0.4127, *P* = 0.0361.

Figure 4d: Percent occupancy of the stimulated bisection of the place preference arena for ChR2-negative controls (n=5) or DAT-ChR2 (n=11) mice receiving SNc stimulation as described in Fig. 4c. Two-way repeated measures ANOVA. Significant effect of genotype, F (1, 14) = 26.41, *P* = 0.0002. Sidak's multiple comparisons post hoc tests. Control vs. DAT-ChR2 (High), *P* = 0.0003. Control vs. DAT-ChR2 (Low), *P* = 0.0111.

Figure 4f: Mean real-time changes in calculated dopamine concentration in dorsal

striatum during 50 Hz 15 mW stimulated trials (red) or non-stimulated trials (black). Time points of significant difference are indicated by the upper thick black line, as measured by Mann-Whitney rank sum test.

Figure 4h: Percent change in the number of presses during stimulation relative to baseline number of presses per sequence for mice receiving 50 Hz 15 mW stimulation. One-way ANOVA, no effect of stimulation position.

Figure 4i: Percent change in sequence stop time following 50 Hz 15 mW stimulation. One-way ANOVA, significant effect of stimulation position, $F(1.876, 16.89) = 25.10$, $P < 0.0001$. Tukey's multiple comparisons post hoc tests. 12 vs 30, $P < 0.0001$. 21 vs 30, $P = 0.0049$.

Figure 4j: Linear regression of the normalized percent reduction in presses and the percent change in stop times for all 50 Hz 15 mW stimulations. Correlation coefficient $r = 0.3005$, ns, $P = 0.1066$.

Figure 4i: Mean real-time changes in calculated dopamine concentration in dorsal striatum during 50 Hz 15 mW stimulated trials (red) or non-stimulated trials (black). Time points of significant difference are indicated by the upper thick black line, as measured by Mann-Whitney rank sum test.

Figure 4n: Percent change in the number of presses during stimulation relative to baseline number of presses per sequence for mice receiving 20 Hz 5 mW stimulation. One-way ANOVA, no significant effect of stimulation position.

Figure 4o: Percent change in sequence stop time following 20 Hz 5 mW stimulation. One-way ANOVA, no significant effect of stimulation position.

Figure 4p: Linear regression of the normalized percent reduction in presses and the percent change in stop times for all 20 Hz 5 mW stimulations. Correlation coefficient r = -

0.07289, ns, $P = 0.7019$.

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