Opponent regulation of action performance and timing by striatonigral and striatopallidal pathways

Authors:

Konstantin I. Bakhurin[1*], Xiaoran Li [1*], Alexander D. Friedman[1], Nicholas A. Lusk[1], Glenn D.R. Watson[1], Namsoo Kim[1], Henry H. Yin[1,2]

Author affiliations:

1. Department of Psychology and Neuroscience, Duke University, Durham, NC, 27708, USA.
2. Department of Neurobiology, Duke University School of Medicine, Durham, NC, 27708, USA.

* These authors contributed equally.

Corresponding author:

Henry H. Yin, Ph.D., hy43@duke.edu

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Abstract

The basal ganglia are widely implicated in action selection and timing, but the relative contributions of the striatonigral (direct) and striatopallidal (indirect) pathways to these functions are still a matter of intense debate. This study investigated the effects of optogenetic stimulation of D1+ (direct) and A2A+ (indirect) neurons in the ventrolateral striatum in head-fixed mice on a fixed time reinforcement schedule. Direct pathway stimulation initiates licking, which can be frequency locked with the laser pulses. Indirect pathway stimulation suppresses non-consummatory licking but elicits rebound licking behavior whose latency is frequency dependent. During peak probe trials that measure the timing of expected reward, direct pathway stimulation reset the internal clock, as measured by the timing of the next bout of anticipatory licking. By contrast, indirect pathway stimulation paused the clock and proportionally delayed the onset of the next bout of licking. Our results provide evidence for the continuous and opposing contributions of the direct and indirect pathways in the production and timing of reward-guided behavior.

Introduction

The striatum is the major input nucleus of the basal ganglia and contains two major populations of medium spiny projection neurons (Gerfen et al., 1990; Mink, 1996). Striatonigral (direct pathway) neurons express D1 receptors and project to the substantia nigra pars reticulata (SNR) and other basal ganglia output nuclei. Striatopallidal (indirect pathway) neurons express D2 receptors and project to the external globus pallidus but is capable of exerting the opposite effect on basal ganglia output nuclei via additional structures. Ever since their basic anatomical connectivity was defined, there has been controversy on their relative contributions to behavior. Given their opposite effects on basal ganglia output, an influential model of the striatum casts the direct and indirect pathways in opposing roles with the direct pathway as an action initiator and the indirect pathway as a braking system (Albin, Young, & Penney, 1989; Freeze, Kravitz, Hammack, Berke, & Kreitzer, 2013; Kravitz et al., 2010). More recently this view has been questioned by studies that described simultaneous activation of these two populations during

behavior (Cui et al., 2013; Isomura et al., 2013) suggesting complementary contributions of these two pathways.

The lateral or sensorimotor striatum receives topographically organized innervation from the cortex and thalamus (Hintiryan et al., 2016). This pattern is also confirmed functionally by the finding that micro-stimulation of different striatal regions results in movements of different body parts (Alexander & DeLong, 1985). Although licking behavior is predominantly controlled by brain stem pattern generators (Travers, Dinardo, & Karimnamazi, 1997), basal ganglia output can provide top-down regulation of licking by influencing the pattern generators, either directly or via the tectum (Deniau & Chevalier, 1992; Redgrave, Marrow, & Dean, 1992; Rossi et al., 2016; Shammah-Lagnado, Costa, & Ricardo, 1992; Toda et al., 2017). In rats, neurons in the ventrolateral striatum correspond to orofacial behaviors, including licking (Mittler, Cho, Peoples, & West, 1994). Stimulation or enhancing dopaminergic signaling in this region can generate orofacial behavior, in some cases dyskinetic movements, while dopamine depletion reduce such behaviors (Delfs & Kelley, 1990; Jicha & Salamone, 1991; Kelley, Lang, & Gauthier, 1988). However, the detailed pathway-specific mechanisms underlying such observations remain largely unknown.

One important characteristic of self-initiated behaviors is that they are often precisely timed relative to some anticipated event like a reward (Gibbon, Church, & Meck, 1984; Pavlov, 1927; Roberts, 1981). The basal ganglia have been repeatedly linked to interval timing in the seconds to minutes range. Manipulation of dopamine activity can bidirectionally influence estimation of the passage of time (Buhusi & Meck, 2005; Meck, 1996) and lesions of the striatum also dramatically impact timing behavior (Meck, 2006). Furthermore, network activity in the striatum has also been shown to reflect the passage of time (Bakhurin et al., 2017; Gouvea et al., 2015). Although the role of D1-expressing and D2-expressing neurons in timing have been interrogated using systemic pharmacological manipulations (Frederick & Allen, 1996), it remains unclear how the two pathways of the basal ganglia interact to mediate these behaviors.

In this study, we used optogenetic stimulation to activate specific cell populations in the direct and indirect pathways of the orofacial striatum during interval timing behavior. Using a fixed-time (FT) schedule of reinforcement, we found that mice rapidly showed highly predictable licking patterns that reflects reliable internal timing processes around reward delivery (Toda et al., 2017). Using temporally and spatially precise manipulations of neural activity with

62 optogenetics, we attempted to determine how different striatal cell populations regulate the
63 initiation, maintenance, and timing of licking behaviors.
64
65 **Results**
66 To study the roles of the direct and indirect pathways during timing behavior, we
67 manipulated the activity of these cell populations in the orofacial region of the striatum (Figures
68 1A & B). We bilaterally injected a Cre-dependent AAV5-DIO-ChR2 virus in the VLS region of
69 the striatum in D1-Cre (Figure 1C) and A2A-Cre (Figure 1D) mice and received chronic optic
70 fiber implants in addition to a head-post for head-fixation. Five additional A2A-Cre mice
71 received AVV5-DIO-eYFP virus. We tracked licking behavior in head-fixed mice performing an
72 interval timing task (Toda et al., 2017). Mice received a 5 μL drop of 10% sucrose solution every
73 10 seconds (Figure 1D). Well-trained animals reliably generated anticipatory licking behavior
74 before reward delivery (Figure 1E). Voluntary licking behavior in all mice consisted bouts of
75 rhythmic protrusion and retractions of the tongue at a relatively fixed rate (4-8 Hz). All mice
76 showed anticipatory licking starting 4-6s before the reward and peaking at roughly the expected
77 time of reward delivery (Figure 1F).
78 We activated the direct pathway in the VLS in well-trained D1-Cre animals at three
79 different time points during the interval. We stimulated the direct pathway for 1 second before
80 animals normally initiate anticipatory licking, starting at 5 seconds before reward delivery
81 (Figure 2A). Though licking was generally sparse at this time point (Figure 2B, top), activation
82 of direct pathway MSNs resulted in large increases in licking rate (Figure 2B, bottom and Figure
83 2C, Video S1). We determined the change in lick rate that resulted from laser stimulation by
84 calculating the lick rate obtained during the 1 second laser stimulation period for D1-Cre mice
85 and a control group that received eYFP virus. The normal lick rate obtained for the same time
86 point during trials without laser stimulation was subtracted from this value to quantify the laser-
87 induced change in lick rate (Figure 2D). A two-way ANOVA revealed a significant change in
88 licking rate by VLS direct pathway stimulation ($F = 56.32$, $p < 0.0001$) in addition to a
89 significant interaction between experimental group and laser frequency ($F = 8.79$, $p < 0.01$). We
90 next stimulated the direct pathway at 1 second prior to reward delivery (Figure 2E), during the
91 time of maximal anticipatory licking (Figure 2F, top). Although there was a ceiling effect on
92 licking at this time point (Figure 2F, bottom), we regularly observed a potentiation of the licking

93 rate during stimulation when compared to trials without stimulation (Figures 2G). A two-way
94 ANOVA revealed significant increase in licking rate as a result of VLS stimulation (Figure 2H,
95 $F = 54.11$, $p < 0.0001$), but no interaction between group and stimulation frequency ($F = 5.94$, p
96 $= 0.16$). In contrast to these effects, stimulation at the time of reward delivery (Figure 2I) had the
97 most modest impact on licking rate (Figures 2J and 2K). A two-way ANOVA detected a
98 significant effect of stimulation on licking even during consumption (Figure 2L, $F = 16.43$, $p <$
99 0.01), but did not show a significant interaction between group and stimulation frequency ($F =$
100 9.88, $p = 0.18$). Together, these results show for the first time that direct pathway stimulation in
101 the orofacial striatum can result in the generation of licking behavior. The effects of direct
102 pathway stimulation were less apparent when we stimulated during on-going anticipatory
103 licking, which can be explained by a ceiling effect.

104 Remarkably, we observed that it was possible for licks to become entrained or time-
105 locked to laser pulses (Figure 3A). We analyzed licking patterns in the frequency domain. While
106 naturally generated licking showed a predominant frequency of 5-9 Hz across animals (Figure
107 3B), licking during laser presentation could be shifted rightward toward a frequency of 10 Hz,
108 which appears to be the upper limit of the central pattern generator for licking (Figure 3C, Video
109 S2). We quantified power spectral density (PSD) distributions for licking and determined the
110 licking frequency that corresponded to the peak of the PSD during laser delivery and during
111 naturally-generated licking without stimulation. Animals displayed two distinct licking
112 frequency levels, depending on whether licking occurred prior to or following reward. Laser
113 stimulation further increased the licking frequency to around 10 Hz, which is rarely reached
114 naturally. Indeed, we found that the peak power of licking was potentiated: there was an
115 interaction between the effects of stimulation frequency and licking type on the peak of the PSD
116 function (Figure 3D, two-way, mixed ANOVA, $F = 12.92$, $p < 0.05$). While we found a
117 significant effect of stimulation on the actual licking frequency (Figure 3E, two-way repeated
118 measures ANOVA, $F = 31.62$, $p < 0.0001$), there was no interaction between frequency and the
119 type of licking ($F = 4.82$, $p = 0.23$). Stimulation also impacted peak PSD power in a similar
120 manner when we stimulated the direct pathway in the middle of the interval at 5 seconds. We
121 found a significant interaction between the effects of stimulation frequency and licking type on
122 peak power (Figure 3F, $F = 13.28$, $p < 0.01$). We also observed a significant effect of stimulation
123 on licking frequency (Figure 3G, $F = 43.22$, $p < 0.0001$), but no significant interaction between

124 stimulation frequency and lick type ($F = 5.34$, $p = 0.1$). An interaction between the effects of
125 licking type and stimulation frequency on peak power was even observed when stimulation
126 occurred during consumption licking (Figure 3H, $F = 9.76$, $p < 0.05$), in addition to an increase
127 in the licking frequency (Figure 5I, $F = 62.35$, $p < 0.001$). Interestingly, 5 Hz stimulation did not
128 reduce the peak licking frequency. Regardless of stimulation frequency, light delivery to the
129 VLS resulted in a positive shift in the frequency domain, above the naturally occurring states that
130 mice normally generate. In addition, direct pathway stimulation could increase the consistency of
131 licking at a given frequency, reflected in the PSD function, suggesting a role for the basal ganglia
132 in controlling the duty cycle of the licking oscillator.

133 When stimulating the direct pathway at 5 seconds prior to reward, we observed that
134 licking resulting from stimulation only began after a certain delay (Figure 4A), and that this
135 delay was reduced with higher stimulation frequencies (Figure 4B). A one-way, repeated
136 measures ANOVA revealed a significant effect of stimulation frequency on the latency to initiate
137 licking following the onset of laser stimulation (Figure 4C; $F = 4.2$, $p < 0.05$). The lick bout that
138 resulted from stimulation also tended to last longer than the 1 second stimulation period. We
139 counted the number of licks that occurred over the course of one second following the offset of
140 stimulation and compared that value with the number of licks normally detected during the same
141 period in non-stimulation trials. Stimulation would result in sustained licking following offset of
142 stimulation, but this was not related to the stimulation frequency (Figure 4D; two-way mixed
143 ANOVA; $F = 12.23$, $p < 0.001$). These results show that greater stimulation of the direct
144 pathway results reduces licking frequency, and that licking continues for a time beyond the
145 immediate period of laser stimulation.

146 The indirect pathway primarily innervates the external globus pallidus, which then sends
147 inhibitory projections to the basal ganglia output neurons in SNR. Activation of the indirect
148 pathway therefore would disinhibit basal ganglia output. We stimulated MSNs expressing ChR2
149 driven by the A2A promoter in the VLS while animals performed the timing task. We found that
150 in contrast to D1 activation, indirect pathway activation resulted in a pronounced suppression of
151 licking. When stimulation occurred 5 seconds prior to reward delivery (Figure 5A), we observed
152 a reduction in licking rate (Figures 5B and 5C). We found a significant effect of experimental
153 group on the reduction in licking rate (Figure 5D, two-way ANOVA, $F = 18.56$, $p < 0.01$), but
154 no interaction between experimental group and frequency ($F = 9.93$, $p = 0.11$). Although we did

155 regularly observe a suppression of licking activity, the likelihood of licking at this period in the
156 interval was low, resulting in a potential floor effect. It was unclear whether indirect pathway
157 activation would as effectively suppress more vigorous licking behavior that is seen prior to
158 reward. When activating the indirect pathway 1 second prior to reward (Figure 5E), we also
159 observed a significant impact on anticipatory licking by indirect pathway stimulation (Figures 5F
160 and 5G, Video S3). In addition to a strong suppression of licking by indirect pathway stimulation
161 (Figure 5H, two-way ANOVA; significant effect of experimental group $F = 25.93$, $p < 0.0001$),
162 we observed a significant interaction between the experimental group and stimulation frequency
163 ($F = 13.83$, $p < 0.001$). We also tested the effect of indirect pathway activation on consumption
164 licking that occurs after reward delivery (Figure 5I). Interestingly, we were less able to suppress
165 licking that occurred following reward (Figures 5J and 5K). Nevertheless there was a significant
166 effect of experimental group on the change in lick rate (Figure 5L, two-way ANOVA, $F = 8.37$,
167 $p < 0.05$), in addition to a significant interaction between indirect pathway activation and licking
168 suppression in the period following reward delivery ($F = 13.08$, $p < 0.05$). Together, these results
169 reveal a frequency-dependent effect of indirect pathway stimulation on licking behavior, which
170 can impact both anticipatory and consumption licking.

171 When we activated the indirect pathway at 5 seconds prior to reward delivery, at the point
172 in the interval when animals are least likely to lick, we observed a striking rebound of licking
173 that followed the offset of stimulation (Figures 6A and B, Video S4). Rebound licking was
174 characterized by higher licking rates during the second following laser offset as compared to the
175 same time point in the absence of stimulation (Figure 6C, two-way repeated measures ANOVA,
176 $F = 11.73$, $p < 0.01$), but there was no interaction between stimulation frequency and the
177 rebound licking rate ($F = 1.16$, $p = 0.78$). We also quantified the latency to lick initiation
178 following laser termination. Indirect pathway stimulation resulted in a reduced latency to initial
179 lick onset following stimulation offset when compared to non-stimulation trials (Figure 6D, two-
180 way RM ANOVA, $F = 16.05$, $p < 0.001$). There was a significant interaction between lick onset
181 latency and the stimulation frequency, with higher frequencies resulting in lower onset latencies
182 ($F = 16.82$, $p < 0.01$). Rebound lick onset latency also showed reduced variance, with higher
183 stimulation frequencies resulting in more consistent rebound latencies (Figure 6E, two-way RM
184 ANOVA, interaction between stimulation frequency and trial type $F = 25.63$, $p < 0.001$).

We observed that the effects of laser stimulation would be altered over the course of the behavioral session, as a function of the animal's motivational state. For example, the effects of direct pathway stimulation at 5 seconds prior to reward (Figure 7A) on changes in lick rate were often observed to be dependent on how many trials the animals received (Figure 7B). At higher frequencies, this gradual reduction of laser-generated licking rate was not as evident (Figure 7C). This could reflect an interaction between the amount of direct pathway recruitment during stimulation and a motivational variable. We divided the session into quarters and quantified the increase of licking rate by stimulation as a function of trial quartile (Figure 7D). A two-way, mixed ANOVA revealed a significant interaction between frequency and quartile ($F = 3.51$, $p < 0.01$) in addition to a significant effect of trial quartile ($F = 1.69$, $p < 0.05$). We also found that the latency to lick onset that results from stimulation also was increased over time. A two-way, mixed ANOVA revealed a significant effect of trial quartile on lick inset latency ($F = 3.03$, $p < 0.05$) but no interaction between trial quartile and stimulation frequency ($F = 1.08$, $p = 0.9$), suggesting a uniform increase in latency across all parameters. Like the effects of stimulation of the direct pathway on lick rate, the latency to rebound licking following stimulation at 5 seconds was sensitive to a motivation variable (Figure 7F). The latency of bound licking increased as the session progressed, but this was most evident at higher stimulation frequencies (Figure 7G, two-way RM ANOVA, interaction between trial quartile and stimulation frequency $F = 8.21$, $p < 0.05$, main effect of trial quartile $F = 5.12$, $p < 0.05$). These results suggest that engagement of lower-level licking generating areas by the basal ganglia can be modulated by motivational signals, but that this impact can be overcome by higher direct pathway activity.

The basal ganglia have been extensively linked to timing, but investigation of the roles of the direct and indirect pathway in mediating timing have not been explored. Our task allowed for the interrogation of the roles of these pathways in timing, as animals will readily anticipate the arrival of the reward (Figure 8A). We incorporated probe trials into the behavioral sessions in which the delivery of a reward was not followed by the expected reward 10 seconds later. Instead, animals generate anticipatory behavior that never encounters a reward delivery (Figure 9B). Because probe trials are not cued, the result is that animals produce a discrete bout of licking with a beginning and an end that reflects the operation of an internal timing process (Roberts, 1981). Prior work manipulating neural function of the basal ganglia has revealed alterations in the peak on probe trials (Meck, 2006). On a fraction of the probe trials we

performed stimulation of the direct and indirect pathways to examine their contributions to timing.

We observed that stimulation of the direct pathway during the probe trial could shift the peak rightward, but in a manner that maintained the interval duration of ~10 seconds following the start of the stimulation. Thus, stimulation of the direct pathway concurrently with reward did not result in an obvious peak shift (Figure 9A, *left*). However, as we moved the stimulation time rightward by 3 or 5 seconds, the peak would move rightward by 3 or 5 seconds, respectively (Figure 10A, *middle* and *right*). Indeed, we found that the peak shift relative to non-stimulation probe trials is a function of the start time of direct pathway stimulation (Figure 10B, one-way, RM ANOVA; $F = 21.68$, $p < 0.001$). Control animals did not display any shifts in peak timing (data not shown; one-way RM ANOVA; $F = 0.7$, $p = 0.5$). These results reveal a critical role of direct pathway activation in resetting the internal timekeeping mechanism. This is the first report of a specific neural correlate of timer resetting. Interestingly, stimulation prior to reward during probe trials did not impact the peak, suggesting that normally reward receipt may be involved in resetting the clock during interval timing on this task.

We also tested the effect of indirect pathway stimulation on timing during probe trials. As observed previously, stimulation of the indirect pathway at 5 seconds after reward resulted in a rebound in licking following the termination of laser. This was also the case during peak probe trials. We found that animals generated 2 unique types of peak responses following rebound licking. We regularly observed that rebound licking would result in a brief delay in licking which was followed by the production of the peak (recovery trials, Figure 10A). In addition, rebound licking could also initiate the peak, appearing to pull the peak towards the rebound effect ('absorb' trials, Figure 10B). Animals would also sometimes not generate rebound licking but would still generate a peak (no-rebound trials, Figure 10C). These three trial types were found to be equally distributed among peak trials that contained laser stimulation across all animals (Figure 10D, $n = 5$, one-way RM ANOVA; $F = 0.2$, $p > 0.05$). Interestingly, peak analysis on these three trial types revealed distinct effects of indirect pathway stimulation on interval timing. For each type of trial, we compared the mean peak time with the peak time on trials in the same session that did not contain laser stimulation. During recovery trials, we found a large rightward shift of the peak that averaged approximately 2 seconds (Figure 10E, two-tailed t-test, $p < 0.01$). In contrast, we found that when licking was absorbed into the rebound licking bout, the peak was

not affected. On trials that lacked rebound licking, we found a rightward peak shift of approximately 750 ms (two-tailed t-test, $p < 0.05$). Although absorb-type trials did not show a change in peak timing, we found that these peaks showed an increased peak duration (Figure 10F, two-tailed t-test, $p < 0.05$).

Finally, we asked if the type of peak trial that we observed following indirect pathway stimulation was altered as the session progressed. We divided the entire session into two halves and asked what proportion of each trial type were found in the first vs. the second half of the session. We found that probability of detecting a recovery-type peak trial was reduced as the session progressed, whereas the likelihood of obtaining trials that lack a rebound increased (Figure 10G, two-way, mixed ANOVA; interaction of the effects of trial type and session half, $F = 25.61$, $p < 0.01$). We did not detect a change in the probability of detecting the absorb-type trials.

Discussion

The striatum is known to play a critical role in governing goal-directed behavior. However, the precise relationships between the direct and indirect pathways that arise from this nucleus are still not well understood. We found for the first time that optogenetic activation of direct-pathway neurons in the ventrolateral striatum can initiate licking (**Figure 2**), whereas activation of the indirect pathway in this region can terminate licking (**Figure 5**).

Our ability to precisely modulate tongue movements in the ventrolateral striatum is in accord with previous work showing relationships between orofacial movements and spiking activity, non-selective electrical stimulation, and dopamine agonist treatment in this region (Mittler et al., 1994; Salamone, Mahan, & Rogers, 1993). While previous studies have showed that activation of the dorsolateral striatum produces locomotion and turning behavior (Bartholomew et al., 2016; Kravitz et al., 2010; Tecuapetla, Matias, Dugue, Mainen, & Costa, 2014), the acute effects on licking in our study is not surprising given the topographical organization of the sensorimotor striatum and our target in the ventrolateral orofacial region. However, we cannot rule out the possibility that the same striatal units can be activated to induce several different behaviors depending on context. As the head-fixed preparation makes it impossible for the other behaviors to be expressed, the trained behavior, namely licking, may become the prioritized output under this condition.

We showed that the orofacial striatum contributes to the top-down initiation and control of licking behavior. Although basal ganglia projections to lower brainstem central pattern generators (CPG) are not required for reflexive orofacial and tongue movements (Grill & Norgren, 1978), an intact basal ganglia are required for goal-directed food seeking behavior (Bignall & Schramm, 1974). It has been shown that a subset of GABAergic projections from the substantia nigra pars reticulata (SNr) to the deep layers of the superior colliculus is critical for the initiation and termination of self-paced licking behavior (Redgrave et al., 1992; Rossi et al., 2016; Toda et al., 2017). A pause in licking can be caused by increased activity in nigrotectal output (Rossi et al., 2016; Toda et al., 2017). As predicted from these anatomical relationships, our experiments show that direct pathway activation may inhibit SNr output and that indirect pathway stimulation may disinhibit nigrotectal inhibitory activity. It is also critical to note that the GABAergic output from the SNr can also directly innervate the reticular formation (Deniau & Chevalier, 1992), regions of which are known to be involved in the generation of licking behavior (Travers et al., 1997).

One limitation of previous studies on functional roles of direct and indirect pathways is inadequate quantification of behaviors. Most spontaneous or trained behavior is complex, sparse or infrequently performed, or can be difficult to quantify. In our approach, animals generate many individual movements that are structured in time around predicted reward delivery and allowed us to better understand the interactions of the direct and indirect pathways in regulating ongoing action, via top-down projections to licking CPGs with some activation threshold. The direct pathway can result in activation above threshold, and the latency of this activation is related to the magnitude of direct pathway activation (stimulation frequency, **Figure 4**). Activity of the indirect pathway shows an opposite effect on licking, in that it can suppress the lick generator, resulting in a pause in behavior.

We revealed several important details about how basal ganglia outputs can modulation of licking by analyzing licking in the frequency domain. We found that licking frequency could enter a high-frequency mode that exceeded the frequency ranges normally observed in mice. We showed for the first time that top-down modulation of central patterns generators takes the form of duty cycle regulation. That is to say, the descending signals from the basal ganglia modulate the ‘on time’ of a relatively stereotyped pattern generator, presumably in the brainstem. Interestingly, we consistently found that these effects saturate above 25 Hz for stimulation of

either pathway. That is, the strongest possible duty cycle modulation (proportion of on time or off for the lick pattern generator) is achieved at this stimulation frequency. On the other hand, the fastest possible rate of licking is around 9-10 Hz, the limit of the lick pattern generator. Normally, the lick pattern generator oscillates at 5-6 Hz for anticipatory licking and 6-8 Hz for consummatory licking. Stronger signals from the direct pathway can increase the lick frequency slightly, but the major effect is to increase the proportion of time that the pattern generator is turned on (duty cycle).

In some cases with 10 Hz stimulation, we could synchronize the lick cycle such that each pulse would be followed by a lick (**Figure 3**). This finding is novel as licking in rodents is well established to have a stereotyped pattern at 6-8 Hz (Travers et al., 1997). This higher-frequency mode was not sensitive to laser stimulation frequency, suggesting the CPG operates in several discrete frequency modes, rather than being able to modulate their output in a continuous manner. This is analogous to a fan with three settings, such as off, low, and high. This parallels the operation of other brain stem systems, such as those regulating locomotion, that also seem to operate in discrete steps or stages (Bellardita & Kiehn, 2015). Together these analyses show that the basal ganglia are involved in regulating the frequency of licking in a step-like manner, and that the basal ganglia output can dynamically control the duty-cycle of lower level CPGs.

We observed a reliable rebound of licking following indirect pathway stimulation in the middle of the interval (**Figure 7**). This was only possible to detect because mice rarely lick at that time point in our task, and the effect was not masked by the delivery of reward or the presence of consumption licking. In addition to the increase in licking rate, we also observed an effect of stimulation frequency on latency to rebound licking (**Figure 6**). Indirect pathway stimulation may result in more active nigral output, which results in a large suppression of downstream structures. The release of this inhibition then gives rise to a burst of rebound activity in regions that are responsible for lick generation, resulting in fast reactivation of licking CPGs above the threshold, and a sudden output of behavior at a consistent latency. The possibility also remains that a large increase in indirect pathway activity results in compensatory activity in the direct pathway, which becomes revealed upon termination of light stimulation, although the results of our peak probe experiments suggest otherwise (see below).

The effects of direct and indirect pathway stimulation were sensitive to a motivational variable, as the latencies to licking, overall licking rates, and the latency to rebound were

340 observed to alter over the course of the session (**Figure 7**). These results can also be explained
341 by the CPG threshold model outlined above: over time, the threshold that must be exceeded to
342 drive licking can be increased, or that the activating signal attenuates as animals become sated.
343 We have previously proposed that the SNr contains output projection neurons that
344 operate in opposition during the deviation of movements away from neutral postures or states
345 (Barter et al., 2015; Fan, Rossi, & Yin, 2012; Yin, 2014). The present results are consistent with
346 the potential role of the direct and indirect pathways for modulating in tandem these opponent
347 output channels. The direct pathway is sufficient for bringing the activity of lick-generating
348 centers above a threshold; however, the indirect pathway may play an important role in
349 maintaining output within a specific range once above that threshold. We could expose the
350 physical limits of the system by selectively driving the direct pathway, though animals rarely
351 enter this regime on their own. Because of the concurrent operation of the indirect pathway,
352 output is rarely expressed at its maximum level. It is only under conditions of artificially
353 targeting these two pathways separately that behavior can be described as operating in an all-or-
354 none, go/no-go fashion.
355 Prior observations of coincident activity of the two pathways may reflect a coordinated
356 regulation of behavior to keep output within a certain range, one that does not always maximize
357 the potential output that is possible by the system. The balanced relationship between these two
358 pathways may be responsible for the continuous adjustments to specific kinematic details of
359 ongoing movement. However, it is unlikely that direct and indirect pathway activity are precisely
360 synchronized as that would simply generate conflict and prevent behavior. Rapid alternation
361 during normal behavior is more likely given our observation of clear opponent effects.
362 Our results also shed light on the distinct contributions of direct and indirect pathways to
363 timing. The basal ganglia have long been implicated in interval timing (Meck, 2006). However,
364 little has been done to explore the relative contributions of the direct and indirect pathways in
365 interval timing (Frederick & Allen, 1996). The use of the fixed-interval schedule in this licking
366 task allowed us to incorporate peak probe trials that enables the investigation of the basal ganglia
367 pathways' contributions to timing of goal-directed behavior.
368 We found that stimulation of the direct pathway reset the interval timing mechanism and
369 activation of the indirect pathway could pause its operation (**Figures 9 & 10**). These
370 observations are in accord with previous pacemaker-accumulator models (Gibbon et al., 1984).

In these models, once a switch is thrown to close the circuit, the accumulator integrates output from a pacemaker. A comparator continuously monitors the difference between the accumulator's integrated value and a stored reference memory for the accumulator's state that corresponds to achieving the trained interval. An approximate difference of zero in the value computed by the comparator results in initiation of responding. The accumulator is then emptied, and the switch is opened until the next stimulus that signals the system to begin counting. The neural implementation of these variables has not been identified. In the present study we found for the first time that direct pathway activity appears to enter an accumulator.

During peak probe trials, mice show an average rate of responding that peaks at the trained interval of 10 seconds. When stimulating the direct pathway, we found that the peak would appear another 10 seconds following the onset of stimulation: The interval was maintained, but the activation of the direct pathway appeared to reinitiate the accumulation of the 10 second interval, like resetting some 'internal clock'.

On the other hand, indirect pathway stimulation did not result in a resetting effect, suggesting again that resetting is specific to direct pathway activation and not a consequence of any distracting neural perturbation. This detail also argues against the possibility that the rebound effect is mediated by overactivated direct pathway activity. We found a complex interaction between the timing system and the rebound licking that immediately follows indirect pathway stimulation. In general, the chief effect of indirect pathway activation is a pause in the timer, similar to what has been observed previously with nigrotectal stimulation. The duration of this pause appeared to shift the peak rightward by at least one second, because we observed a significant delay in peak time (approximately 750 ms) during trials that contained stimulation but did not contain rebound licking. In addition, the presence of the rebound added an additional second to the peak's shift, suggesting that rebound licking is also pauses the accumulator. This impact on timing was only observed occasionally, as sometimes rebound licking simply initiated the peak. These trials were also noteworthy in that they resulted in a peak of longer duration, but not one with a significant leftward shift, indicating that timing was not perturbed on these trials, only the initiation of the bout. Distinct types of trials observed could be due to variable rates of filling the accumulator: faster filling on a given trial would allow the rebound licking to initiate the peak, whereas slower filling of the accumulator would mean that indirect pathway activity or a resulting rebound would only interrupt or pause this process. In partial support of this idea are

402 our observations of the motivational effects on behavioral responses to indirect pathway
403 stimulation: rebound licking latency becomes greater over the course of the session and that the
404 proportion of overall peak-probe trials that contained rebound licking was reduced over session
405 time.
406 In summary, we have shown for the first time that direct pathway stimulation in the
407 ventrolateral striatum can initiate licking, whereas indirect pathway stimulation can suppress
408 ongoing licking. Direct pathway stimulation is capable of driving licking behavior beyond the
409 limits of normal behavior. This suggests that these pathways can work in concert to continuously
410 regulate the magnitude and frequency ongoing licking. Lastly, the distinct effects of direct and
411 indirect stimulation on timing behavior suggest that each pathway differentially interacts with the
412 internal time-keeping system: direct pathway stimulation can discharge the accumulator, whereas
413 indirect pathway activation can only pause or interfere with the accumulation process. These
414 principles may be extended to explaining how the basal ganglia regulate volitional actions in
415 general.
416
417
418 **Methods**
419
420 *Subjects:* 8 adult D1-Cre and 13 A2A-Cre mice of both sexes were used (Jackson Labs, Bar
421 Harbor, ME). These mice expressed Cre-recombinase only in D1 or A2A striatal neurons,
422 respectively. All experiments were conducted during the light phase of the animals' 12:12 light
423 cycle. Mice had unrestricted access to food in their home cages, but were water deprived and
424 received a 10% sucrose solution during behavioral sessions. Animal weight was maintained at
425 85% body weight and was monitored on a daily basis, with additional water given after
426 experimental training as needed. All experimental procedures were approved by the Duke
427 University Institutional Animal Care and Use Committee.
428
429 *Viral vectors*: AAV5-EF1α-DIO-hChR2(H134R)-eYFP was injected into 8 D1-Cre and 8 A2A-
430 Cre mice. AAV5-EF1α-DIO-eYFP was injected into 5 A2A-Cre mice for control experiments.
431 Vectors were obtained from the University of North Carolina Vector Core.
432

Surgery: Mice were placed in a stereotaxic frame and anesthetized with ~1-1.5% isoflurane. Small holes were drilled bilaterally at AP: +0.2 mm and ML: +/- 2.5 mm relative to bregma. Stereotaxically guided injections of (200 nL per hemisphere) were made at AP: +0.2 and ML: 2.5 and DV: -4.6 & -4.4 relative to bregma using a Nanoject III microinjector (Drummond Scientific, Broomall, PA). 100 µm-core optic fibers (Precision Fiber Products, Chula Vista, CA) were implanted with the same AP and ML coordinates and at DV: -4.2 in order to target cell bodies of the VLS. Fibers were secured to the skull with screws and dental cement along with a head post for head fixation.

System for behavioral training and recording: Mice were allowed 7-14 days to recover from surgery and then were water deprived in their home cages. The interval timing task is the same as previously described (Toda et al., 2017). Briefly, behavioral experiments were conducted in a sound-proof chamber with the animals perched in a custom-made elevated tunnel platform. Animals' heads were clamped in place on either side of the head using the implanted steel head post. A metal drinking spout was positioned directly in front of the mouth of the animal so that the animal only needs to protrude its tongue to access the spout. A 10% sucrose solution was gravity fed to the spout and its delivery was controlled by the opening of a solenoid valve. The spout was connected to a capacitance touch-sensor (MPR121, Adafruit, New York, NY) coupled to an Arduino Leonardo (www.arduino.cc) that reported the time of each contact with the spout by the tongue. The task was controlled using custom Matlab (version 2014b, Mathworks, Natick, MA) programs interfaced with a Blackrock Cerebrus recording system (Blackrock Microsystems, Salt Lake City, UT) to generate digital signals to control reward delivery, and analog commands to drive laser stimulation. The Cerebrus system was simultaneously used for recording these signals in addition to the digital timestamps received from the lickometer. All timestamps were saved for offline analysis.

Fixed-interval timing task: Initial training began with water-deprived mice and involved habituation to head-fixation and training animals to collect experimenter-delivered water drops from the spout. Once reliable licking was established for drop delivery after a few days, training began on a fixed-interval version of the task. During the task, animals received a 5 µL drop of reward every 10 seconds. No external stimuli were presented. White noise was played inside of

the chamber to mask the sound of the solenoid (Rossi & Yin, 2015). Water was presented at fixed intervals for 200 trials a day until animals showed anticipatory licking behavior.

Peak probe trials: Once animals displayed consistent anticipatory licking in during fixed-interval sessions, sessions began to incorporate peak probe trials. During peak trials, no reward is presented in order to measure the accuracy of animals' internal timing ability. Peak probe trials occurred with a 60% possibility after 3 consecutive rewards. No indication was given as to when a peak trial would occur. Peak trials lasted 30 seconds plus a random duration sampled from a gamma distribution (Matlab function *gamrnd* using shape parameter = 2.5 and scale parameter = 4). The consecutive reward counter was then reset with a reward delivery. Animals that struggled to learn this version of the peak procedure were run on a schedule that used a 30% probability of peak probe trial occurrence after 3 consecutive rewards.

Optogenetic stimulation: Optical stimulation occurred both in rewarded and probe trials to prevent mice from predicting the probe trials with the sensation of the stimulation. Stimulation on a given trial was determined using random sampling from a uniform distribution. Following 3 fixed-interval trials without laser delivery, the program entered a decision point. There was a 30% chance of laser delivery during a normal trial, a 30% chance of laser delivery during a peak trial, and a 30% chance of a peak trial without laser. The remaining 10% of cases resulted in a new decision point. This resulted in a 10-15% of all trials being a peak trial without stimulation, a peak trial with stimulation, or a normal. Once head fixed in the testing chamber, mice were connected bilaterally to a 473-nm DPSS laser (BL473T3, Shanghai Laser, Shanghai, China) via fiber optic cables. A fiber splitter (TM105R5F1A, ThorLabs, Newton, NJ) divided the beam (50:50) for bilateral stimulation. Stimulation was pulsed (0.8-2mW; 5-50 Hz, 10 ms square pulse width, 1 s duration). Stimulation onset was either at -1 s from reward, was concurrent with the time of reward (0s condition) or occurred 5 s prior to the reward. During peak probe trials, stimulation was delivered at -1 s from reward, with the time of reward, or 5 seconds following reward delivery. Stimulation parameters (stimulation frequency and timing relative to reward) were consistent within a session, but the order of stimulation was semi-randomized between mice.

Quantification and Statistical Analysis: Behavioral data was analyzed with NeuroExplorer (Nex Technologies, Colorado Springs, CO) and Matlab. Licking timestamps were filtered to exclude events occurring less than 8 ms apart. Unless otherwise stated, all analyses were performed on the first half of trials to exclude motivational effects. Statistical tests were performed in GraphPad Prism (GraphPad Software, San Diego, CA). Two-tailed or one-tailed parametric tests were used. Power spectrum analysis was performed in NeuroExplorer.

Peak analysis: Peak-detection and quantification was performed on a single-trial basis using a custom-written Matlab algorithm. For each peak probe trial, licking time stamps were aligned to the reward that initiated the trial. An analysis window extending 0 to 20 seconds following the reward was used to detect peaks. Licking within this window was binned using 100ms time bins to produce a continuous licking rate estimate and smoothed using a gaussian filter with a width of 1 second. The Matlab function *findchangepts* was then applied to the smoothed lick rate signal to detect steep transitions in licking rate. These change points were classified as rising or falling transitions based on the slope of the lick rate signal around them. The first increasing change point in the analysis window and the next falling change point were determined to represent the start and end of the peak, respectively. The peak was fit with a gaussian function, the maximum of which was taken as the peak for that trial. Peak duration had to be at least 2 seconds in duration and licking rate had to exceed 3 Hz to be included. In cases of failures to identify a peak, the analysis window was shifted by +500 milliseconds and the procedure was applied again up to a maximum of 5 shifts, after which the trial would be discarded upon failure to detect a valid peak. To detect peaks occurring after direct pathway stimulation at 3 or 5 seconds, the 20 second analysis window was shifted right by 3 or 5 seconds, respectively, following the reward to exclude stimulation-evoked licking as a peak.

Lick onset latency analysis: Lick onsets was defined as the first lick occurring after all inter-lick-intervals of 1 second or greater. For determining the latency to lick onset, the first lick onset occurring during the 1 second laser stimulation was counted for each stimulation trial. For determining rebound licking onset time, we measured the time until the first lick onset that occurred within a window of 0 to 3 seconds following the offset of laser termination. The same window was applied to non-laser trials for within-subject comparisons. Trials without licking

were excluded from analysis.

Post rebound peak trial classification: Peak trials that contained indirect pathway activation at 5 seconds post reward were classified by the temporal relationships of lick onsets relative to rebound licking. Trials that contained a lick onset in a 2 second period following stimulation onset were considered having a rebound. Otherwise, the trial was classified as lacking a rebound. For trials classified as a rebound-containing peak trial, a second lick onset timestamp had to occur within 8 seconds following initiation of rebound licking. In this case, the trial was classified as a rebound recovery trial. Otherwise, if no lick onset timestamp followed the rebound, the trial was classified as a rebound absorption trial. To detect peaks during rebound recovery trials, the analysis window was shifted right by 6 seconds from reward delivery in order to exclude the rebound from being counted as a peak.

Author contributions:

Author contributions: XL and HHY designed research; KIB, XL, ADF, NAL, GDRW performed research; KIB, XL, ADF, NAL, and HHY analyzed data; and KIB, and HHY wrote the paper.

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Competing interests:

The authors declare no competing financial interests.

References:

Albin, R. L., Young, A. B., & Penney, J. B. (1989). The functional anatomy of basal ganglia disorders. *Trends Neurosci, 12*(10), 366-375.

Alexander, G. E., & DeLong, M. R. (1985). Microstimulation of the primate neostriatum. II. Somatotopic organization of striatal microexcitable zones and their relation to neuronal response properties. *J Neurophysiol, 53*(6), 1417-1430.

Bakhurin, K. I., Goudar, V., Shobe, J. L., Claar, L. D., Buonomano, D. V., & Masmanidis, S. C. (2017). Differential Encoding of Time by Prefrontal and Striatal Network Dynamics. *J Neurosci, 37*(4), 854-870.

Barter, J. W., Li, S., Sukharnikova, T., Rossi, M. A., Bartholomew, R. A., & Yin, H. H. (2015). Basal ganglia outputs map instantaneous position coordinates during behavior. *J Neurosci, 35*(6), 2703-2716.

Bartholomew, R. A., Li, H., Gaidis, E. J., Stackmann, M., Shoemaker, C. T., Rossi, M. A., & Yin, H. H. (2016). Striatonigral control of movement velocity in mice. *Eur J Neurosci, 43*(8), 1097-1110.

Bellardita, C., & Kiehn, O. (2015). Phenotypic characterization of speed-associated gait changes in mice reveals modular organization of locomotor networks. *Curr Biol, 25*(11), 1426-1436.

Bignall, K. E., & Schramm, L. (1974). Behavior of chronically decerebrated kittens. *Exp Neurol, 42*(3), 519-531.

Buhusi, C. V., & Meck, W. H. (2005). What makes us tick? Functional and neural mechanisms of interval timing. *Nat Rev Neurosci*.

Cui, G., Jun, S. B., Jin, X., Pham, M. D., Vogel, S. S., Lovinger, D. M., & Costa, R. M. (2013). Concurrent activation of striatal direct and indirect pathways during action initiation. *Nature, 494*(7436), 238-242.

Delfs, J., & Kelley, A. (1990). The role of D 1 and D 2 dopamine receptors in oral stereotypy induced by dopaminergic stimulation of the ventrolateral striatum. *Neuroscience, 39*(1), 59-67.

Deniau, J. M., & Chevalier, G. (1992). The lamellar organization of the rat substantia nigra pars reticulata: distribution of projection neurons. *Neuroscience, 46*(2), 361-377.

Fan, D., Rossi, M. A., & Yin, H. H. (2012). Mechanisms of action selection and timing in substantia nigra neurons. *J Neurosci, 32*(16), 5534-5548.

Frederick, D. L., & Allen, J. D. (1996). Effects of selective dopamine D1- and D2-agonists and antagonists on timing performance in rats. *Pharmacol Biochem Behav, 53*(4), 759-764.

Freeze, B. S., Kravitz, A. V., Hammack, N., Berke, J. D., & Kreitzer, A. C. (2013). Control of basal ganglia output by direct and indirect pathway projection neurons. *J Neurosci, 33*(47), 18531-18539.

Gerfen, C. R., Engber, T. M., Mahan, L. C., Susel, Z., Chase, T. N., Monsma, F. J., Jr., & Sibley, D. R. (1990). D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science, 250*(4986), 1429-1432.

Gibbon, J., Church, R. M., & Meck, W. H. (1984). Scalar timing in memory. *Ann N Y Acad Sci, 423*, 52-77.

Gouvea, T. S., Monteiro, T., Motiwala, A., Soares, S., Machens, C., & Paton, J. J. (2015). Striatal dynamics explain duration judgments. *Elife, 4*.

Grill, H. J., & Norgren, R. (1978). The taste reactivity test. II. Mimetic responses to gustatory stimuli in chronic thalamic and chronic decerebrate rats. *Brain Res, 143*(2), 281-297.

Hikosaka, O. (2007). GABAergic output of the basal ganglia. *Prog Brain Res, 160*, 209-226.

Hintiryan, H., Foster, N. N., Bowman, I., Bay, M., Song, M. Y., Gou, L., . . . Dong, H. W. (2016). The mouse cortico-striatal projectome. *Nat Neurosci, 19*(8), 1100-1114.

Isomura, Y., Takekawa, T., Harukuni, R., Handa, T., Aizawa, H., Takada, M., & Fukai, T. (2013). Reward-modulated motor information in identified striatum neurons. *J Neurosci, 33*(25), 10209-10220.

Jicha, G. A., & Salamone, J. D. (1991). Vacuous jaw movements and feeding deficits in rats with ventrolateral striatal dopamine depletion: possible relation to parkinsonian symptoms. *J Neurosci, 11*(12), 3822-3829.

Kelley, A. E., Lang, C. G., & Gauthier, A. M. (1988). Induction of oral stereotypy following amphetamine microinjection into a discrete subregion of the striatum. *Psychopharmacology (Berl), 95*(4), 556-559.

Kravitz, A. V., Freeze, B. S., Parker, P. R., Kay, K., Thwin, M. T., Deisseroth, K., & Kreitzer, A. C. (2010). Regulation of parkinsonian motor behaviours by optogenetic control of basal ganglia circuitry. *Nature, 466*(7306), 622-626.

Meck, W. H. (1996). Neuropharmacology of timing and time perception. *Brain Res Cogn Brain Res, 3*(3-4), 227-242.

Meck, W. H. (2006). Neuroanatomical localization of an internal clock: a functional link between mesolimbic, nigrostriatal, and mesocortical dopaminergic systems. *Brain Res, 1109*(1), 93-107.

Mink, J. W. (1996). The basal ganglia: focused selection and inhibition of competing motor programs. *Prog Neurobiol, 50*(4), 381-425.

Mittler, T., Cho, J., Peoples, L. L., & West, M. O. (1994). Representation of the body in the lateral striatum of the freely moving rat: single neurons related to licking. *Exp Brain Res, 98*(1), 163-167.

Pavlov, I. (1927). *Conditioned Reflexes*. Oxford: Oxford University Press.
Redgrave, P., Marrow, L., & Dean, P. (1992). Topographical organization of the nigrotectal projection in rat: evidence for segregated channels. *Neuroscience, 50*(3), 571-595.
Roberts, S. (1981). Isolation of an internal clock. *J Exp Psychol Anim Behav Process, 7*(3), 242-268.
Rossi, M. A., Li, H. E., Lu, D., Kim, I. H., Bartholomew, R. A., Gaidis, E., . . . Yin, H. H. (2016). A GABAergic nigrotectal pathway for coordination of drinking behavior. *Nat Neurosci*.
Rossi, M. A., & Yin, H. H. (2015). Elevated dopamine alters consummatory pattern generation and increases behavioral variability during learning. *Front Integr Neurosci, 9*, 37.
Salamone, J. D., Mahan, K., & Rogers, S. (1993). Ventrolateral striatal dopamine depletions impair feeding and food handling in rats. *Pharmacol Biochem Behav, 44*(3), 605-610.
Shammah-Lagnado, S. J., Costa, M. S., & Ricardo, J. A. (1992). Afferent connections of the parvocellular reticular formation: a horseradish peroxidase study in the rat. *Neuroscience, 50*(2), 403-425.
Tecuapetla, F., Matias, S., Dugue, G. P., Mainen, Z. F., & Costa, R. M. (2014). Balanced activity in basal ganglia projection pathways is critical for contraversive movements. *Nat Commun, 5*, 4315.
Toda, K., Lusk, N. A., Watson, G. D. R., Kim, N., Lu, D., Li, H. E., . . . Yin, H. H. (2017). Nigrotectal Stimulation Stops Interval Timing in Mice. *Curr Biol, 27*(24), 3763-3770 e3763.
Travers, J. B., Dinardo, L. A., & Karimnamazi, H. (1997). Motor and premotor mechanisms of licking. *Neuroscience & Biobehavioral Reviews, 21*(5), 631-647.
Yin, H. H. (2014). Action, time and the basal ganglia. *Philos Trans R Soc Lond B Biol Sci, 369*(1637), 20120473.

Figure 1

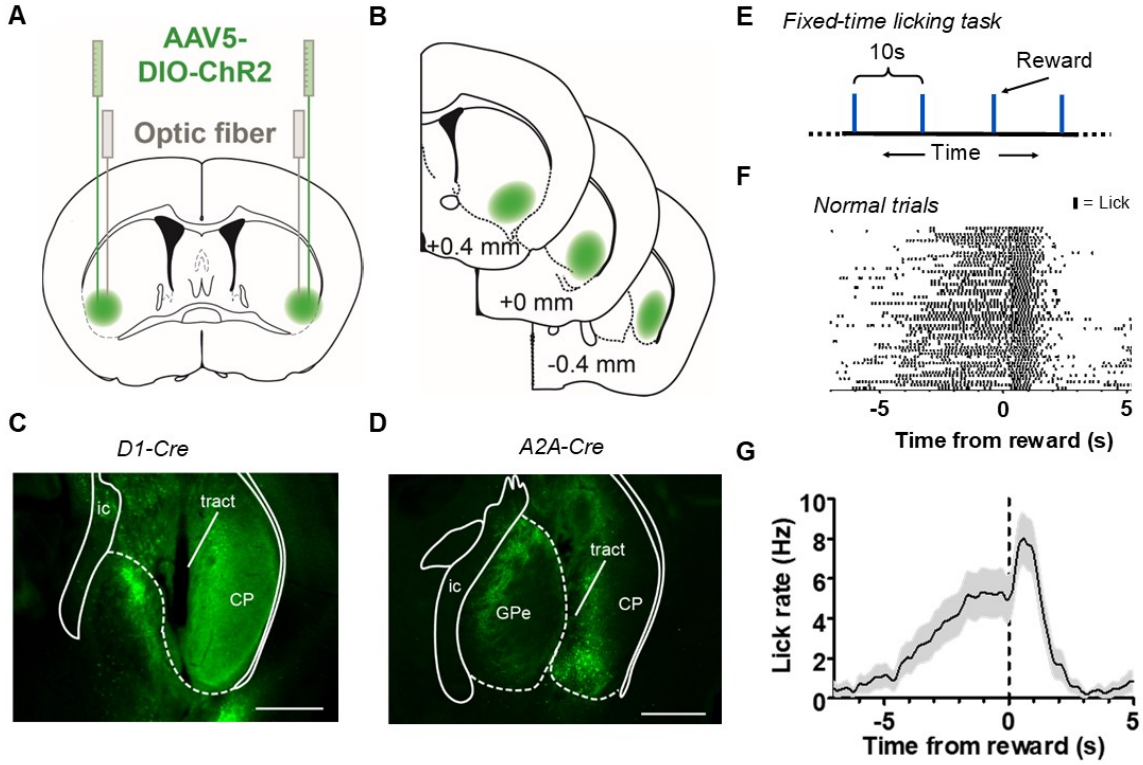
A
AAV5-
DIO-ChR2
Optic fiber
B
+0.4 mm
+0 mm
-0.4 mm
C
D1-Cre
ic
tract
CP
D
A2A-Cre
ic
GPe
tract
CP
E
Fixed-time licking task
10s
Reward
Time
F
Normal trials
= Lick
-5
0
5
Time from reward (s)
G
Lick rate (Hz)
10
8
6
4
2
0
-5
0
5
Time from reward (s)

Figure 2

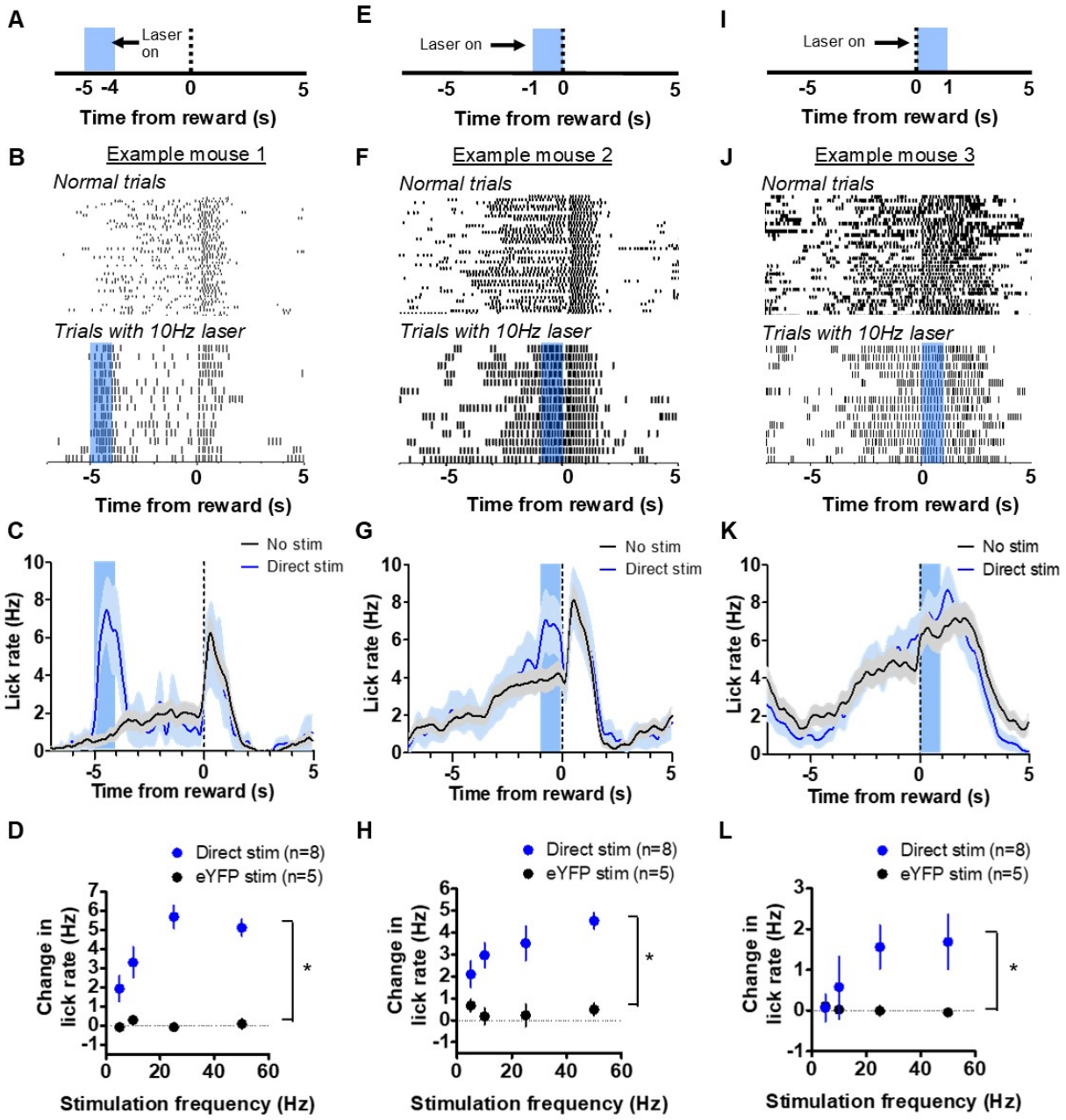
A
Laser on
-5 -4 0 5
Time from reward (s)
E
Laser on
-5 -1 0 5
Time from reward (s)
I
Laser on
-5 0 1 5
Time from reward (s)
B
Example mouse 1
Normal trials
Trials with 10Hz laser
-5 0 5
Time from reward (s)
F
Example mouse 2
Normal trials
Trials with 10Hz laser
-5 0 5
Time from reward (s)
J
Example mouse 3
Normal trials
Trials with 10Hz laser
-5 0 5
Time from reward (s)
C
No stim
Direct stim
Lick rate (Hz)
Time from reward (s)
G
No stim
Direct stim
Lick rate (Hz)
Time from reward (s)
K
No stim
Direct stim
Lick rate (Hz)
Time from reward (s)
D
Direct stim (n=8)
eYFP stim (n=5)
Change in lick rate (Hz)
*
Stimulation frequency (Hz)
H
Direct stim (n=8)
eYFP stim (n=5)
Change in lick rate (Hz)
*
Stimulation frequency (Hz)
L
Direct stim (n=8)
eYFP stim (n=5)
Change in lick rate (Hz)
*
Stimulation frequency (Hz)

Figure 3

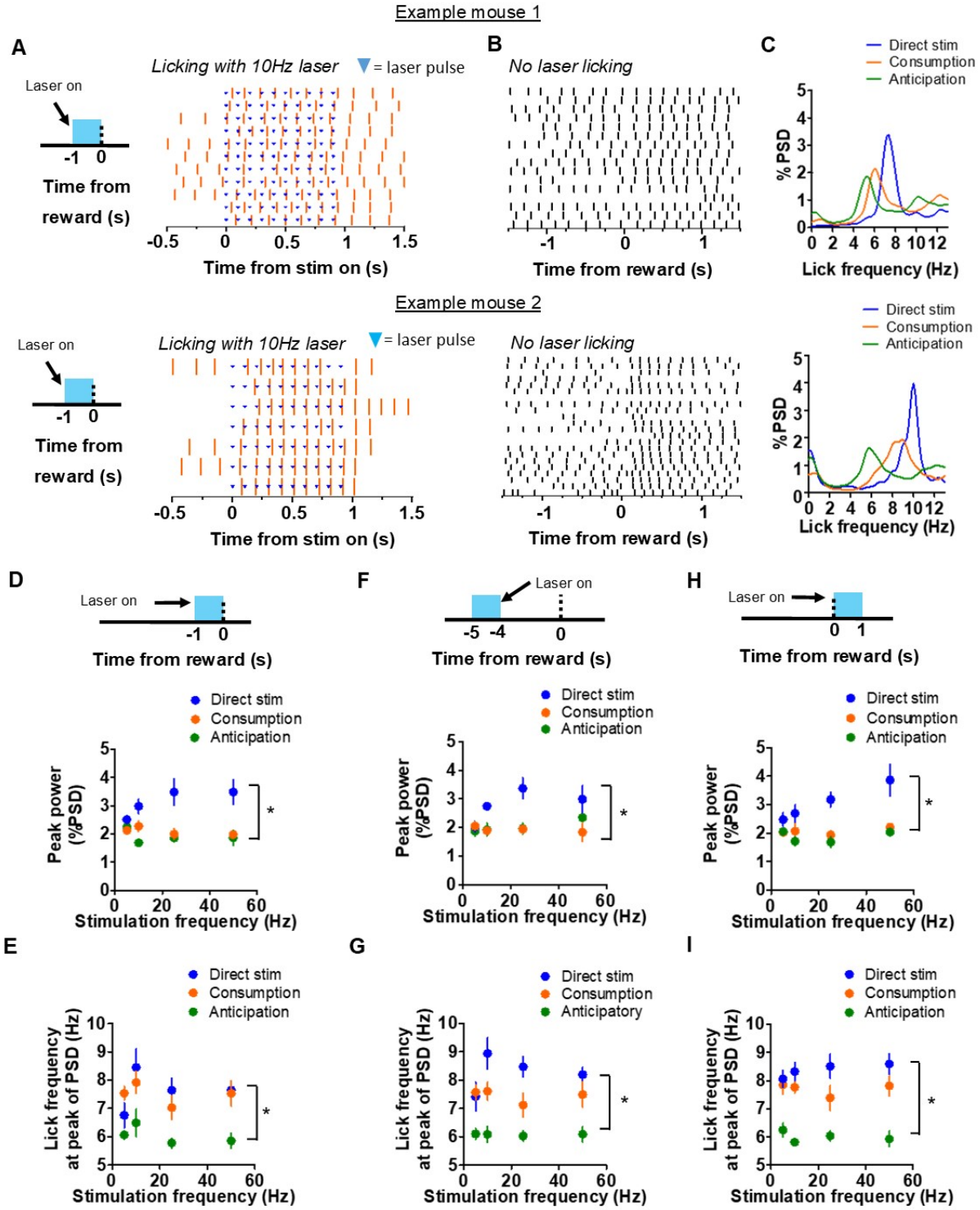
Example mouse 1
A
Laser on
-1 0
Time from reward (s)
Licking with 10Hz laser
= laser pulse
-0.5 0 0.5 1 1.5
Time from stim on (s)
B
No laser licking
-1 0 1
Time from reward (s)
C
Direct stim
Consumption
Anticipation
% PSD
0 2 4 6 8 10 12
Lick frequency (Hz)
Example mouse 2
Laser on
-1 0
Time from reward (s)
Licking with 10Hz laser
= laser pulse
-0.5 0 0.5 1 1.5
Time from stim on (s)
No laser licking
-1 0 1
Time from reward (s)
Direct stim
Consumption
Anticipation
% PSD
0 2 4 6 8 10 12
Lick frequency (Hz)
D
Laser on
-1 0
Time from reward (s)
Direct stim
Consumption
Anticipation
Peak power (%PSD)
Stimulation frequency (Hz)
F
Laser on
-5 -4 0
Time from reward (s)
Direct stim
Consumption
Anticipation
Peak power (%PSD)
Stimulation frequency (Hz)
H
Laser on
0 1
Time from reward (s)
Direct stim
Consumption
Anticipation
Peak power (%PSD)
Stimulation frequency (Hz)
E
Direct stim
Consumption
Anticipation
Lick frequency at peak of PSD (Hz)
Stimulation frequency (Hz)
G
Direct stim
Consumption
Anticipatory
Lick frequency at peak of PSD (Hz)
Stimulation frequency (Hz)
I
Direct stim
Consumption
Anticipation
Lick frequency at peak of PSD (Hz)
Stimulation frequency (Hz)

Figure 4

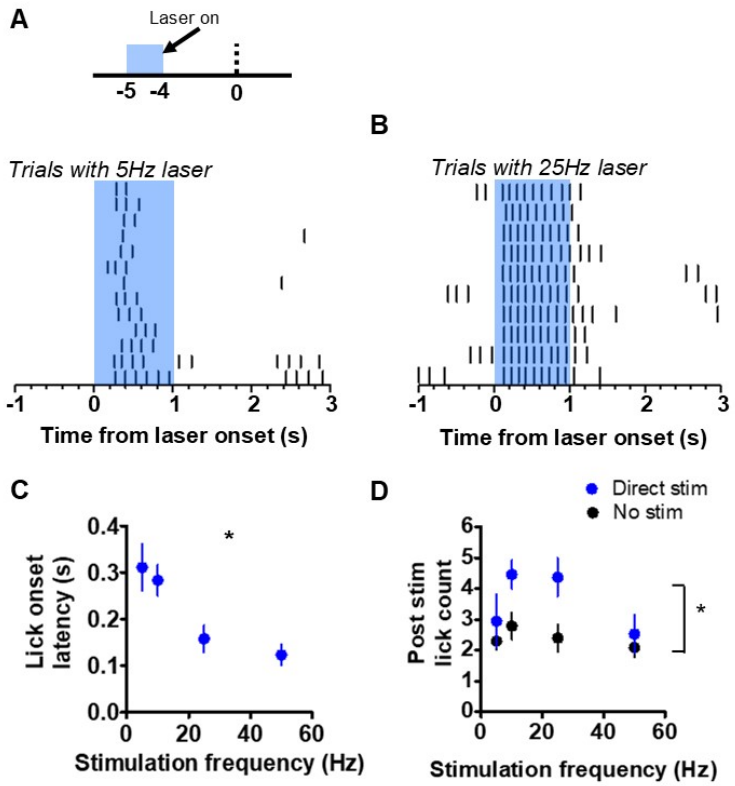
A
Laser on
-5
-4
0
B
Trials with 5Hz laser
Trials with 25Hz laser
-1
0
1
2
3
Time from laser onset (s)
C
D
Direct stim
No stim
Lick onset latency (s)
0.4
0.3
0.2
0.1
0.0
*
0
20
40
60
Stimulation frequency (Hz)
Post stim lick count
6
5
4
3
2
1
0
*

Figure 5

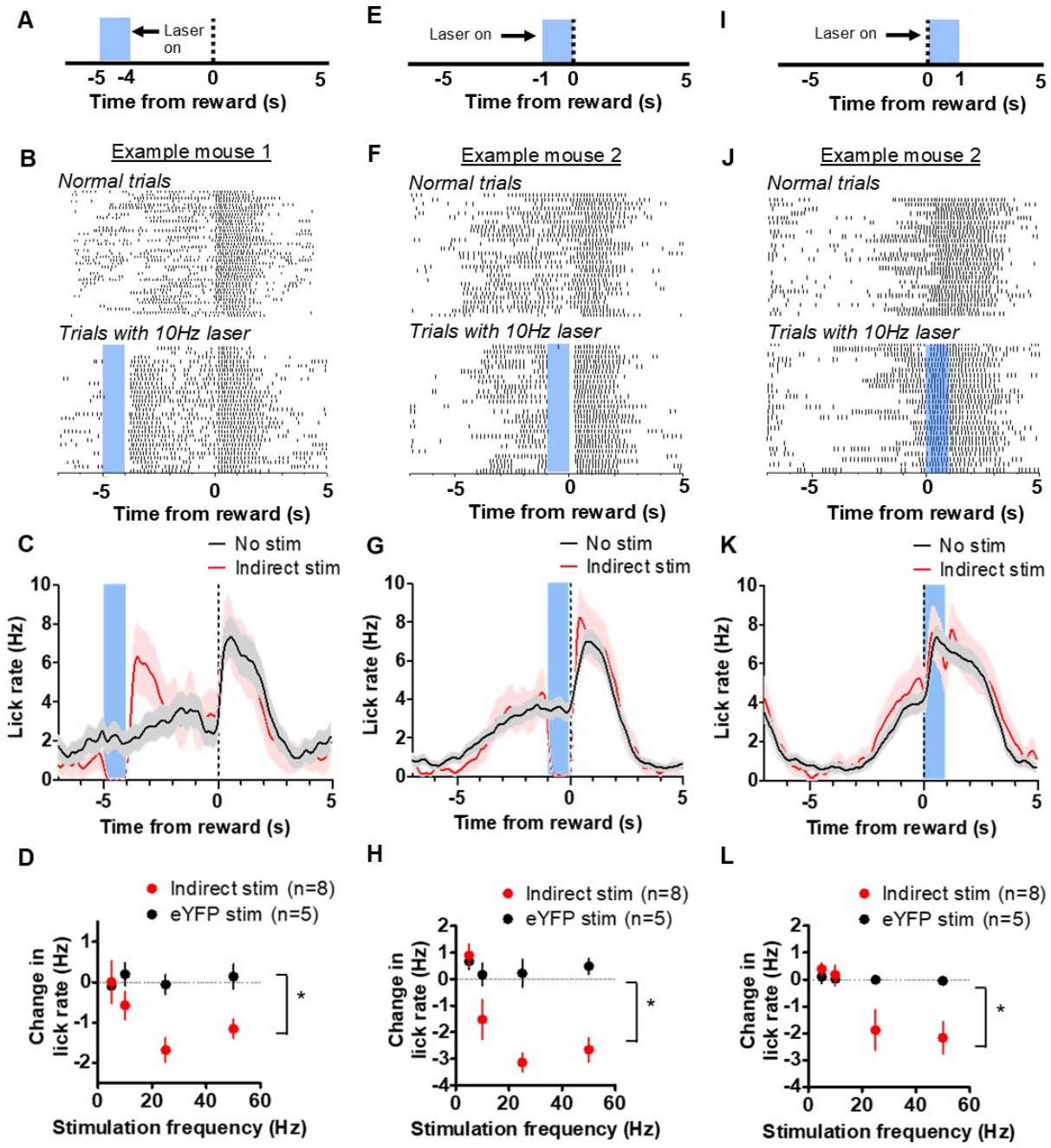
A
Laser on
-5 -4 0 5
Time from reward (s)
E
Laser on
-5 -1 0 5
Time from reward (s)
I
Laser on
-5 0 1 5
Time from reward (s)
B
Example mouse 1
Normal trials
Trials with 10Hz laser
-5 0 5
Time from reward (s)
F
Example mouse 2
Normal trials
Trials with 10Hz laser
-5 0 5
Time from reward (s)
J
Example mouse 2
Normal trials
Trials with 10Hz laser
-5 0 5
Time from reward (s)
C
No stim
Indirect stim
Lick rate (Hz)
Time from reward (s)
G
No stim
Indirect stim
Lick rate (Hz)
Time from reward (s)
K
No stim
Indirect stim
Lick rate (Hz)
Time from reward (s)
D
Indirect stim (n=8)
eYFP stim (n=5)
Change in lick rate (Hz)
Stimulation frequency (Hz)
H
Indirect stim (n=8)
eYFP stim (n=5)
Change in lick rate (Hz)
Stimulation frequency (Hz)
L
Indirect stim (n=8)
eYFP stim (n=5)
Change in lick rate (Hz)
Stimulation frequency (Hz)

Figure 6

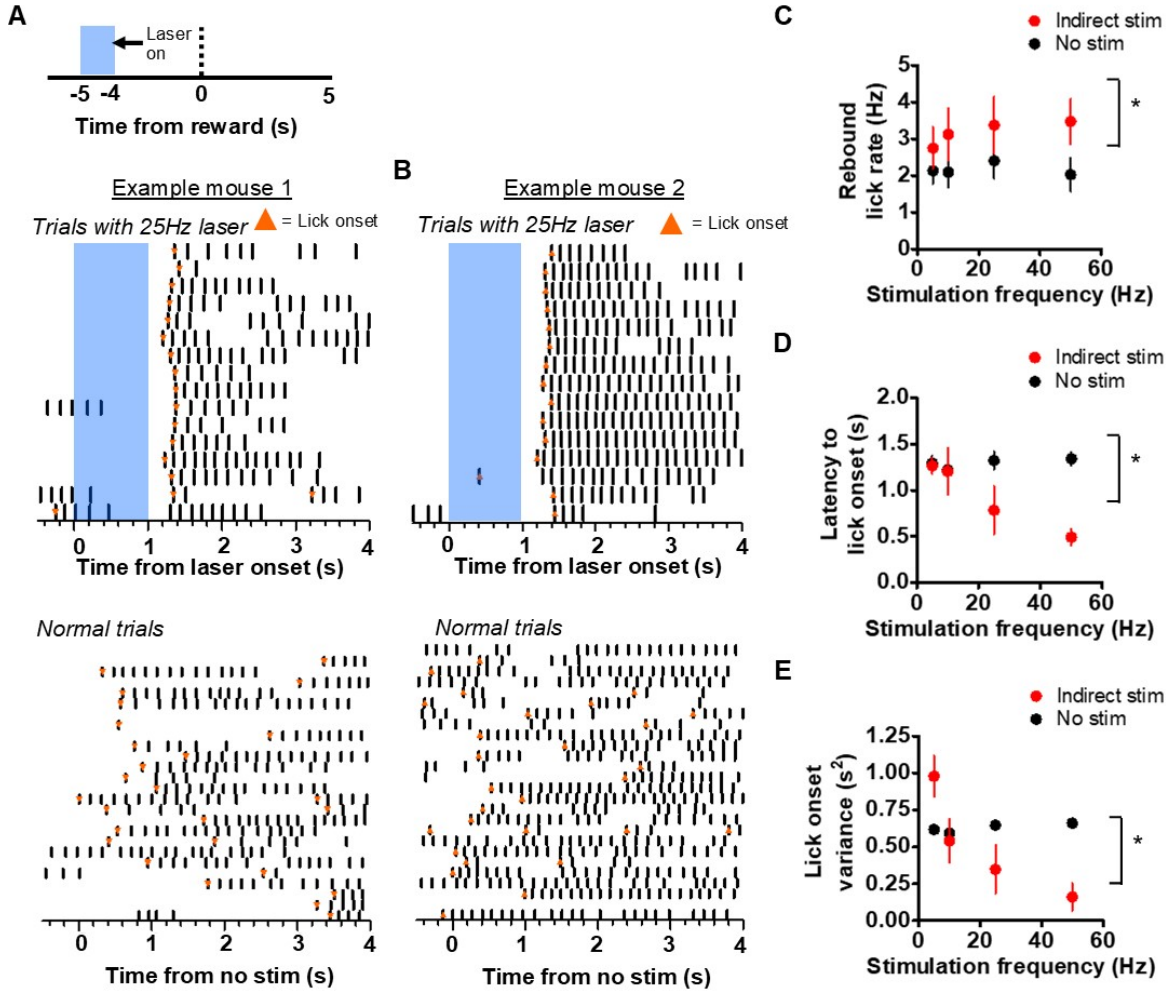
A
Laser on
-5 -4 0 5
Time from reward (s)
B
Example mouse 1
Trials with 25Hz laser
= Lick onset
Time from laser onset (s)
Normal trials
Time from no stim (s)
Example mouse 2
Trials with 25Hz laser
= Lick onset
Time from laser onset (s)
Normal trials
Time from no stim (s)
C
Indirect stim
No stim
Rebound lick rate (Hz)
Stimulation frequency (Hz)
D
Indirect stim
No stim
Latency to lick onset (s)
Stimulation frequency (Hz)
E
Indirect stim
No stim
Lick onset variance (s²)
Stimulation frequency (Hz)

Figure 7

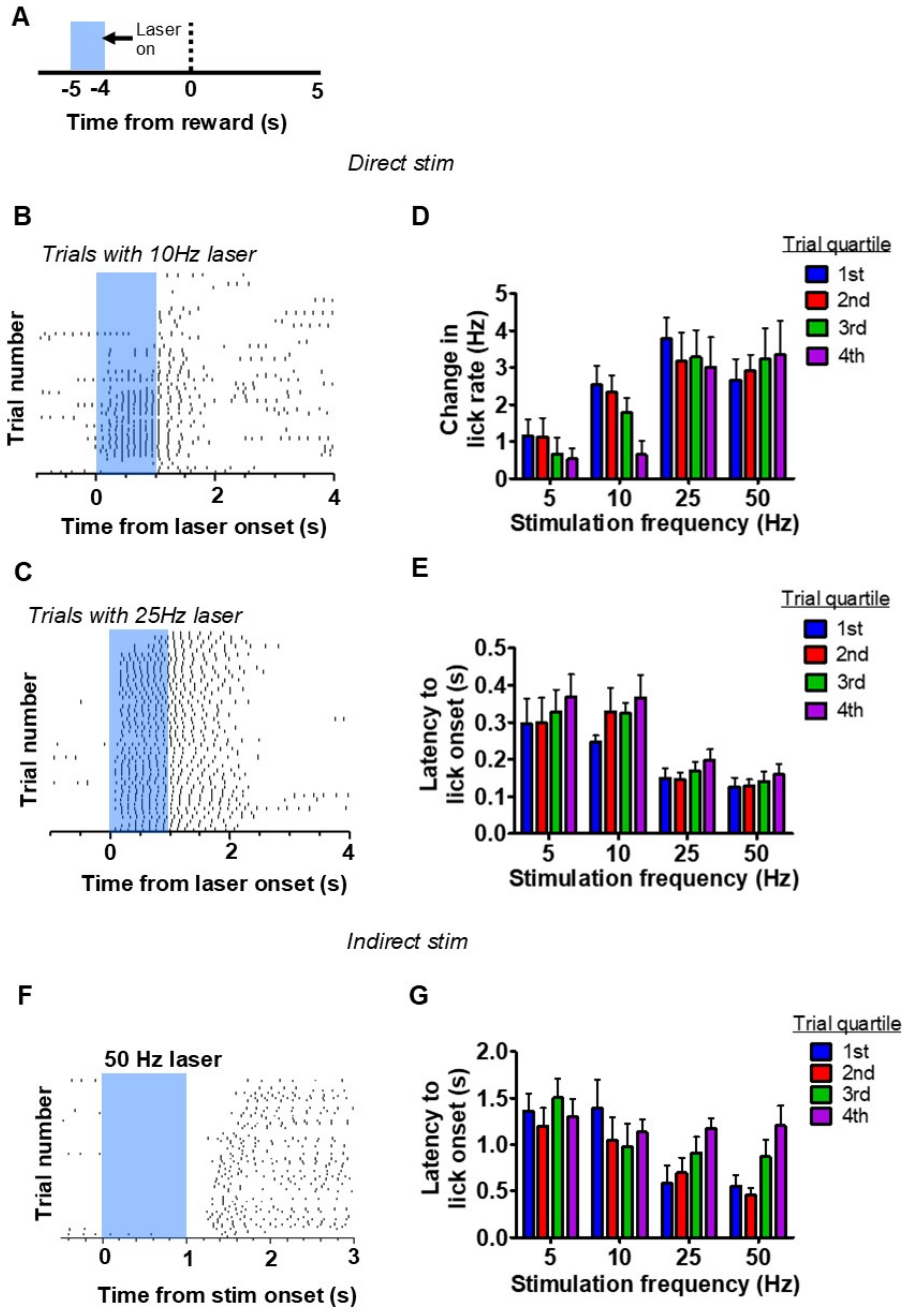
A
Laser on
-5 -4 0 5
Time from reward (s)
Direct stim
B
Trials with 10Hz laser
Trial number
0 2 4
Time from laser onset (s)
C
Trials with 25Hz laser
Trial number
0 2 4
Time from laser onset (s)
D
Trial quartile
1st
2nd
3rd
4th
Change in lick rate (Hz)
0 1 2 3 4 5
5 10 25 50
Stimulation frequency (Hz)
E
Trial quartile
1st
2nd
3rd
4th
Latency to lick onset (s)
0.0 0.1 0.2 0.3 0.4 0.5
5 10 25 50
Stimulation frequency (Hz)
Indirect stim
F
50 Hz laser
Trial number
0 1 2 3
Time from stim onset (s)
G
Trial quartile
1st
2nd
3rd
4th
Latency to lick onset (s)
0.0 0.5 1.0 1.5 2.0
5 10 25 50
Stimulation frequency (Hz)

Figure 8

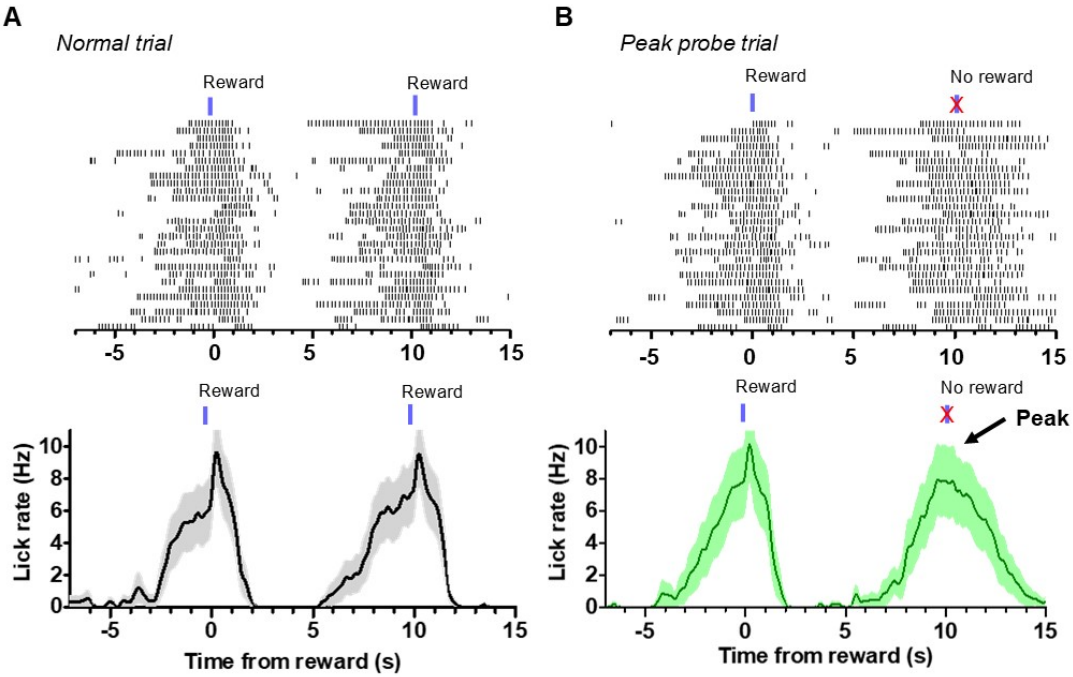
A
Normal trial
Reward
Reward
-5
0
5
10
15
Reward
Reward
Lick rate (Hz)
10
8
6
4
2
0
-5
0
5
10
15
Time from reward (s)
B
Peak probe trial
Reward
No reward
-5
0
5
10
15
Reward
No reward
Peak
Lick rate (Hz)
10
8
6
4
2
0
-5
0
5
10
15
Time from reward (s)

Figure 9

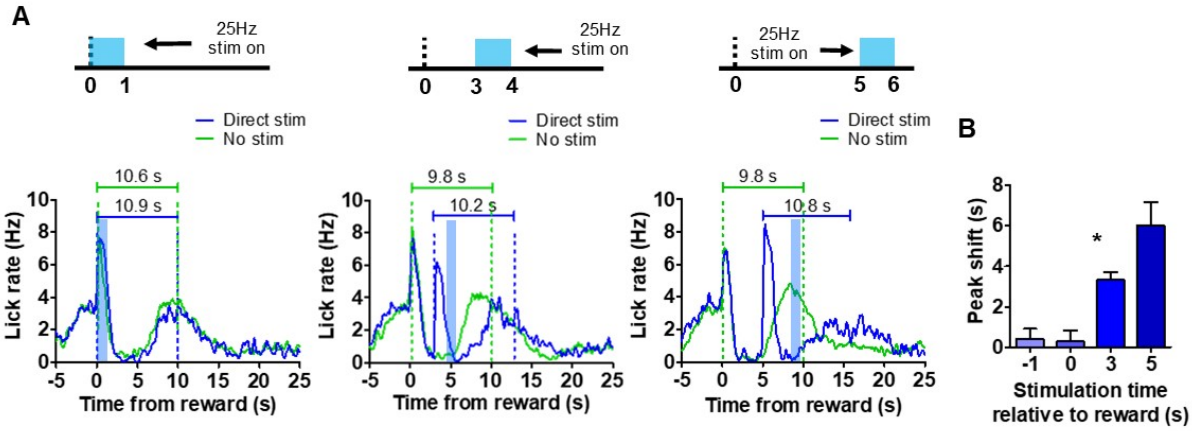
A
25Hz
stim on
0 1
0 3 4
0 5 6
Direct stim
No stim
10.6 s
10.9 s
9.8 s
10.2 s
9.8 s
10.8 s
Lick rate (Hz)
Time from reward (s)
B
Peak shift (s)
*
-1 0 3 5
Stimulation time
relative to reward (s)

Figure 10

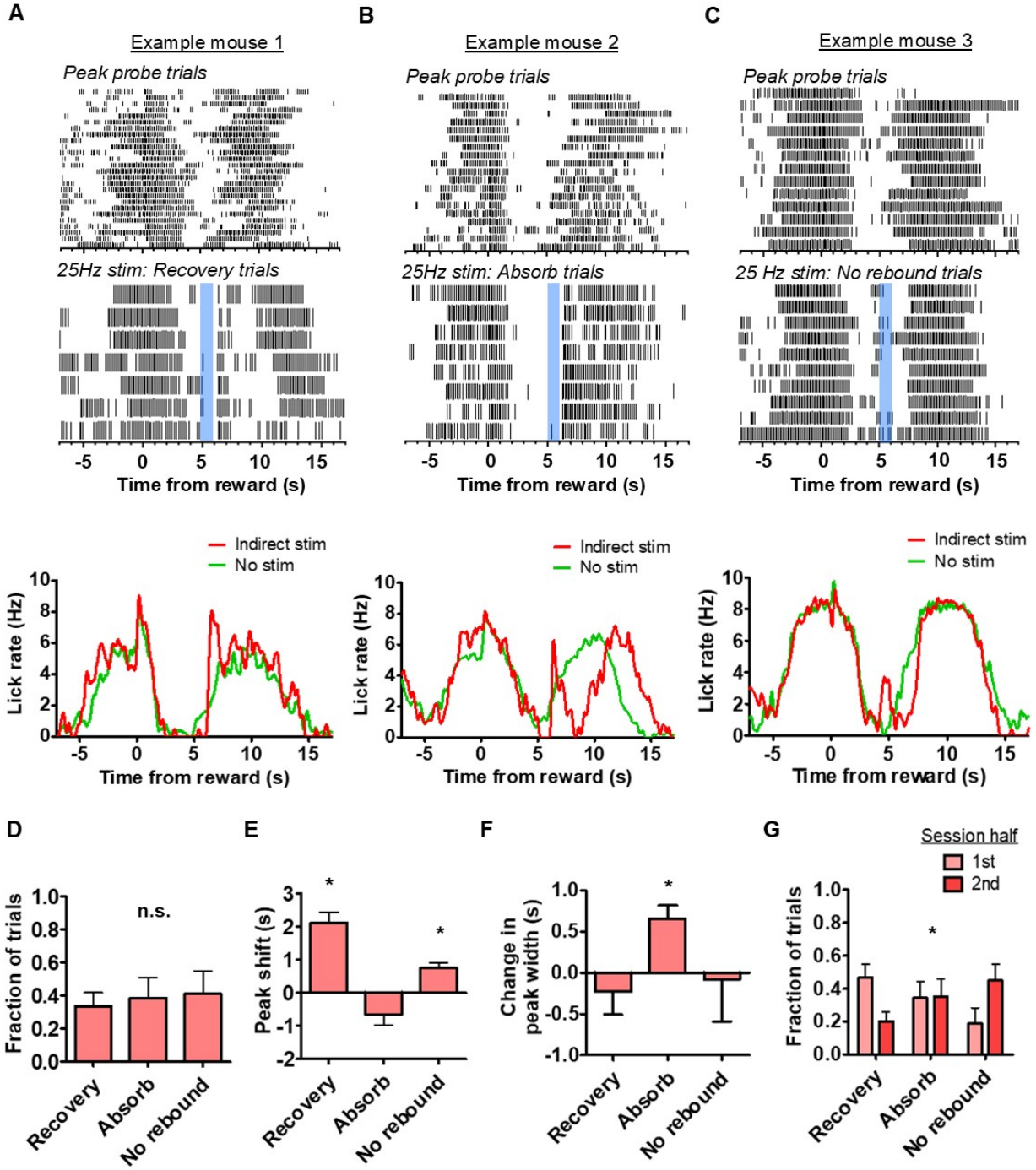
A
Example mouse 1
Peak probe trials
25Hz stim: Recovery trials
Time from reward (s)
B
Example mouse 2
Peak probe trials
25Hz stim: Absorb trials
Time from reward (s)
C
Example mouse 3
Peak probe trials
25 Hz stim: No rebound trials
Time from reward (s)
Indirect stim
No stim
Lick rate (Hz)
Time from reward (s)
D
Fraction of trials
n.s.
Recovery
Absorb
No rebound
E
Peak shift (s)
F
Change in peak width (s)
G
Session half
1st
2nd
Fraction of trials

Figure legends

Figure 1. Stimulation of the direct and indirect pathways of the orofacial striatum during a licking-based interval timing task

A) *Left*: Strategy of injection of AAV-ChR2 virus to orofacial area of the striatum in D1-Cre or A2A-Cre mice and implantation of chronic optical fibers over the injection site. **B**) Serial section diagrams showing target zone for orofacial striatum. **C**) Representative image of eYFP-expression in ventrolateral striatum in a D1-Cre+ mouse injected with AAV-DIO-ChR2-eYFP. Scale bar = 500 µm. **D**) Representative image of eYFP-expression in an A2A-Cre+ mouse injected with AAV-DIO-ChR2-eYFP virus. Scale bars = 500 µm. **E**) Diagram illustrating the fixed-time interval task for head-fixed animals. Mice receive a 10% sucrose reward (5 µL) every 10 seconds. **F**) Example lick raster of a well-trained animal during normal trials aligned to reward delivery. Tick marks represent individual licks. **G**) Mean licking rate calculated from the raster in *E*. Error denotes SEM.

Figure 2. Stimulation of direct pathway in VLS generates licking

A) Depiction of stimulation strategy 5 seconds prior to reward in D1-cre mice. **B**) *Top*: Peri-event lick raster diagrams of representative trials demonstrating normal anticipatory licking patterns in trials without laser. *Bottom*:10 Hz stimulation at 5 seconds prior to reward results in large increases in licking activity. Both rasters reflect licking in the same animal in the same session. **C**) Mean lick rate calculated from the example session shown in *B* for trials with and without laser stimulation. **D**) The mean licking rate during laser delivery resulting from stimulation of D1 MSNs at 5 seconds increased as a function of stimulation frequency (n = 8 mice; two-way ANOVA; main effect of stimulation: $F = 56.4$, $p < 0.0001$; interaction between frequency and group: $F = 8.76$, $p < 0.01$). **E**) Depiction of stimulation strategy 1 second prior to reward. **F**) *Top*: Peri-event lick raster diagrams of representative non-laser trials. *Bottom*: Raster diagram of trials containing 10 Hz stimulation during anticipatory licking. Both rasters reflect licking in the same animal in the same session. **G**) Mean lick rate calculated from the example session shown in *F* for trials with and without laser stimulation. **H**) Stimulation of D1 MSNs at 1 second prior to reward increased the rate of anticipatory licking (two-way ANOVA, main effect of group: $F = 54.11$, $p < 0.0001$). **I**) Depiction of stimulation strategy coinciding with reward delivery. **J**) *Top*: Representative lick raster diagrams of licking behavior during trials without laser stimulation. *Bottom*: Raster diagram of trials from the same animal containing 10 Hz stimulation during consumption licking. Both rasters reflect behavior recorded in the same session. **K**) Mean licking rate calculated from the example session shown in *J* for trials with and without stimulation. **L**) Stimulation of D1 MSNs was capable of increasing consumption licking rate (two-way ANOVA, main effect of group: $F = 16.43$, $p < 0.01$). Error denotes SEM.

Figure 3. Direct pathway boosts licking frequency and modulates licking duty cycle

A) Example lick rasters from two animals showing licking frequency potentiation by direct pathway stimulation 1 second before reward. Note entrainment of licking to 10 Hz stimulation in

the lower plot. Blue tick-marks denote laser pulse times. **B**) Example lick raster from the same sessions as in *A* showing the licking activity around reward. **C**) Demonstration of spectral density analyses of licking during laser stimulation, anticipation (1 second intervals beginning prior to reward), and consumption (1 second intervals for 1 second following reward) periods. Analysis of data from the sessions represented in *A* and *B* is shown. **D**) Effect of stimulation frequency on peak power of the PSD when stimulation occurred during anticipatory licking. Peak licking power was related to stimulation frequency (n = 8 mice; two-way mixed ANOVA; main effect of Lick class, $F = 48.01$, $p < 0.0001$; interaction Lick class vs Frequency, $F = 12.92$, $p < 0.05$). **E**) Mean licking frequency corresponding to the peak of the PSD as a function of laser frequency for stimulation during anticipatory licking. Stimulation increased the licking frequency (two-way mixed ANOVA; $F = 31.62$, $p < 0.0001$). **F**) Effect of stimulation frequency on peak power of licking when stimulation occurred 5 seconds before reward. Peak power was related to stimulation frequency (n = 8 mice, two-way mixed ANOVA; main effect of Lick class, $F = 25.9$, $p < 0.0001$; interaction Lick class vs Frequency, $F = 13.28$, $p < 0.01$). **G**) Mean licking frequency corresponding to the peak of the PSD as a function of laser stimulation frequency delivered 5 seconds prior to reward. Stimulation increased the licking frequency (two-way mixed ANOVA; $F = 43.22$, $p < 0.0001$). **H**) Effect of stimulation frequency on peak power of licking when stimulation occurred during consumption licking. Peak licking power was related to stimulation frequency (n = 8 mice, two-way mixed ANOVA; main effect of Lick class, $F = 44.1$, $p < 0.0001$; interaction Stimulation vs Frequency, $F = 9.76$, $p < 0.05$). **I**) Mean licking frequency corresponding to the peak of the PSD as a function of laser frequency for stimulation during consumption licking. Stimulation increased the licking frequency (two-way mixed ANOVA; $F = 62.35$, $p < 0.0001$). Error bars reflect SEM.

Figure 4. Direct pathway activates modulates onset latency and duration of evoked licking bout

A) *Top*: The effect of stimulation frequency on evoked licking was measured for sessions with stimulation occurring at 5 seconds prior to reward. *Bottom*: Example raster plot showing longer latency to lick and shorter bout duration during 5 Hz direct pathway stimulation. **B**) Stimulation of the direct pathway at 25 Hz resulted in a lasting effect of licking following offset of laser stimulation. **C**) Latency to lick bout onset as a function of the frequency of direct pathway stimulation (n = 8; one-way ANOVA; $F = 4.92$, $p < 0.01$). **D**) Number of licks that were counted during the 1 second following direct pathway stimulation offset. Blue points reflect counts during trials with laser stimulation at 5 seconds before reward. Black points reflect the baseline count number observed during trials without stimulation for the same time period of the interval (two-way mixed ANOVA; main effect of stimulation: $F = 12.23$, $p < 0.001$). Error bars denote SEM.

Figure 5. Stimulation of the indirect pathway in the orofacial striatum suppresses anticipatory licking

A) Depiction of stimulation strategy 5 seconds prior to reward in A2A-cre mice. **B**) *Top*: Peri-event lick raster diagrams of representative trials demonstrating normal anticipatory licking

patterns in trials without laser. *Bottom*:10 Hz stimulation at 5 seconds prior to reward results in a pause in licking. Both rasters reflect licking in the same animal in the same session. **C**) Mean lick rate calculated from the example session shown in *B* for trials with and without laser stimulation. **D**) Reduction of mean licking rate during stimulation of the indirect pathway at 5 seconds (n = 8 mice; two-way ANOVA; main effect of Stimulation, $F = 18.56$, $p < 0.01$). **E**) Depiction of stimulation strategy 1 second prior to reward. **F**) *Top*: Peri-event lick raster diagrams of representative non-laser trials. *Bottom*: Raster diagram of trials containing 10 Hz stimulation of the indirection pathway during anticipatory licking. Both rasters reflect licking in the same animal in the same session. **G**) Mean lick rate calculated from the example session shown in *F* for trials with and without laser stimulation. **H**) Increasing stimulation frequency of the indirect pathway at 1 second prior to reward reduced the rate of anticipatory licking in A2A-Cre mice (two-way ANOVA; main effect of Stimulation, $F = 25.93$, $p < 0.0001$; main effect of Stimulation, $F = 19.47$, $p < 0.001$; interaction Frequency vs Stimulation, $F = 13.83$, $p < 0.01$). **I**) Depiction of stimulation strategy coinciding with reward delivery. **J**) *Top*: Representative lick raster diagrams of licking behavior during trials without laser stimulation. *Bottom*: Raster diagram of trials from the same animal containing 10 Hz stimulation during consumption licking. Both rasters reflect behavior recorded in the same session. **K**) Mean licking rate calculated from the example session shown in *J* for trials with and without stimulation. **L**) Indirect pathway stimulation at the time of reward reduced licking rate (two-way ANOVA; main effect of Stimulation, $F = 8.37$, $p < 0.05$; main effect of Frequency, $F = 15.52$, $p < 0.05$; interaction Stimulation vs Frequency, $F = 13.08$, $p < 0.01$). Error reflects SEM.

Figure 6. Rebound licking following stimulation of the indirect pathway
A) *Top:* Schematic showing stimulation of A2A-Cre mice 5 seconds prior to reward. *Middle*: Example raster plot showing the resulting rebound licking following the termination of stimulation of the indirect pathway. Orange markers indicate the onset time of discrete lick bouts. *Bottom*: Raster plot of licking during trials in the same animal and the same session that did not include stimulation, aligned to the same time point in the interval as above. Note the predominant alignment of lick onset times following laser stimulation. **B**) *Top*: Example of second animal showing rebound licking after indirect pathway stimulation. *Bottom*: Raster plot of licking during non-stimulation trials for the same animal in the same session. **C**) Comparison of licking rate during stimulation and non-stimulation trials. Indirect pathway stimulation resulted in an increase in licking rate following laser termination when compared to the same time period in non-stimulation trials (n = 8 mice; two-way mixed ANOVA, effect of stimulation $F = 11.73$, $p < 0.01$). **D**) Bar plot showing the reduction of lick onset latency following stimulation termination compared to mean latency for the same time period in non-stimulation trials (two-way mixed ANOVA, main effect of stimulation: $F = 16.05$, $p < 0.001$; stimulation x trial type, $F = 16.82$, $p < 0.01$). **E**) Bar plot showing the reduction of lick onset latency variance following stimulation offset compared to the variance of licking initiation for the same time period in non-stimulation trials (two-way mixed ANOVA, main effect of frequency, $F = 20.66$, $p < 0.01$; stimulation x trial type, $F = 25.63$, $p < 0.001$). Error bars denote SEM.

Figure 7. The effect of motivation on licking affected by direct and indirect pathway stimulation

A) The effect of motivation on licking related to direct and indirect pathway manipulation was measured for sessions with stimulation occurring at 5 seconds prior to reward. **B**) Example raster plot showing progressive reduction of licking evoked by 10 Hz direct pathway stimulation at 5 seconds before reward. **C**) Example raster plot showing a reduced influence of motivation on evoked licking with higher frequency stimulation of the direct pathway. Data is from the same animal but different session as shown in *B*. **D**) Grouped bar plot showing the reduced impact of laser stimulation on evoked licking as a function of trial number (n = 8; two-way mixed ANOVA; effect of trial quartile, $F = 1.69$, $p < 0.05$; effect of stimulation frequency, $F = 31.13$, $p < 0.01$; interaction quartile x frequency, $F = 3.51$, $p < 0.01$). Error bars reflect SEM.
E) Grouped bar plot showing an increase of lick onset latency as a result of direct pathway stimulation as a function of trial number (n = 8, two-way mixed ANOVA, effect of trial quartile, $F = 3.03$, $p < 0.05$; effect of stimulation frequency, $F = 35.34$, $p < 0.01$; interaction quartile x frequency, $F = 1.08$, $p = 0.93$). **F)** Example raster plot showing the increase in rebound latency following 50Hz stimulation of the indirect pathway over the course of the experimental session. **G**) Grouped bar plot showing the increase of lick onset latency following laser stimulation offset as a function of trial number (n = 8, two-way mixed ANOVA, effect of trial quartile, $F = 5.21$, $p < 0.05$; effect of stimulation frequency, $F = 18.18$, $p < 0.05$; interaction quartile x frequency, $F = 8.21$, $p < 0.05$). Error bars reflect SEM.

Figure 8. Peak probe trials reveal the operation of an internal timing mechanism

A) *Top*: Example raster plot of licking during consecutive normal 10 second fixed-time trials. Licking is aligned to the first of two consecutive reward delivery times. *Bottom*: Mean licking rate for the session shown in the above raster. **B**) *Top*: Example raster plot showing licking during peak probe trials. During peak trials, reward is delivered then withheld 10 seconds later, resulting in a discrete bout of licking in the absence of any stimuli. *Bottom*: The average licking rate for the session shown in the above raster. Averaging across probe trials results in a characteristic peak in licking. Data shown for both trial types were recorded from the same animal and session. Error reflects SEM.

Figure 9. Direct pathway stimulation resets the internal clock

A) *Left*: Mean licking rate across subjects during peak probe trials with and without laser stimulation concurrent with reward delivery. Scale bar reflects the population mean peak time for probe trails with (blue) and without (green) laser stimulation. *Middle*: Mean licking rate across subjects during probe trials delivered in the presence and absence of laser stimulation. Scale bar reflects the population mean peak time for probe trails with (blue) and without (green) laser stimulation. *Right*: Mean licking rate across subjects during normal probe trials and peak probe trials containing laser stimulation at 5 seconds following reward. Scale bar reflects the population mean peak time for probe trails with (blue) and without (green) laser stimulation. **B**) Magnitudes of peak shifts in seconds as a function of time of direct pathway stimulation. The y-axis reflects the difference between the mean peak time occurring during laser trials subtracted by the mean peak time during trials without stimulation (n = 4; One-way RM ANOVA; $F = 21.68$, $p < 0.001$). Peak analyses were performed on the entire behavioral session to maximize statistical power. Error bars denote SEM.

Figure 10. Indirect pathway stimulation pauses the internal clock

A) *Top*: Peri-event lick raster of a different subject's representative peak probe trials without laser delivery. *Middle*: 25 Hz stimulation at 5 seconds following reward during trials in which rebound licking causes a delay in peak licking. Both rasters reflect licking in the same animal in the same session. *Bottom*: Mean lick rate calculated from the example session shown above for peak probe trials without laser stimulation and those classified as peak "recovery" trials. **B**) *Top*: Peri-event lick raster diagrams of representative peak probe trials without laser delivery demonstrating normal peak licking patterns. *Middle*: 25 Hz stimulation at 5 seconds following reward during trials in which rebound licking attracts peak licking. Both rasters reflect licking in the same animal in the same session. *Bottom*: Mean lick rate calculated from the example session shown above for peak probe trials without laser stimulation and those classified as "absorb" trials. **C**) *Top*: Peri-event lick raster of a third subject's representative peak probe trials produced in the absence of laser delivery. *Middle*: 25 Hz stimulation at 5 seconds following reward during trials in which no rebound licking was detected. Both rasters reflect licking in the same animal in the same session. *Bottom*: Mean lick rate calculated from the example session shown above for peak probe trials without laser stimulation and those classified as "no rebound" trials. **D**) The three types of trial classifications occurred with equal probability throughout the behavioral session ($n = 5$; one-way RM ANOVA, $F = 0.08$, $p = 0.92$). **E**) Peak shifts were measured by subtracting the mean peak time of each type of stimulation trial by the mean peak time for each animal in the absence of stimulation delivery. Recovery and no rebound trials showed significant positive peak shifts (two-tailed t-tests, $p < 0.05$), whereas absorb trials trended toward negative peak shifts (two-tailed t-test, $p = 0.1$). **F**) Absorb trials showed significant increases in the duration of the peak (two-tailed t-test, $p < 0.05$). **G**) Fraction of peak trials with laser stimulation belonging to each trial classification during the first and second halves of the behavioral session. Recovery trials were gradually replaced with trials that did not contain rebound licking (two-way, mixed ANOVA, interaction trial type x session half, $F = 25.61$, $p < 0.01$). Peak analyses were performed on the entire behavioral session to maximize statistical power. Error bars denote SEM.