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

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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 licking and results in rebound licking immediately after the end of stimulation. During peak probe trials that measure the timing of expected reward, direct pathway stimulation produced a resetting of the internal timing process, as measured by timing of the next bout of anticipatory licking relative to time of stimulation. By contrast, indirect pathway stimulation transiently paused the timing mechanism, and proportionally delayed 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 can exert 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, however, 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, combined with occasional peak probe trials, 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 optogenetics, we attempted to determine how different striatal cell populations regulate the initiation, maintenance, and timing of licking behaviors.

Results

To study the roles of the direct and indirect pathways during timing behavior, we manipulated the activity of cell populations that originate these pathways in the orofacial region of the striatum (Figures 1A & B). We bilaterally injected a Cre-dependent AAV5-DIO-ChR2 virus in the VLS region of the striatum in D1-Cre (Figure 1C) and A2A-Cre (Figure 1D) mice and implanted chronic optic fibers above this area in addition to a head-post for head-fixation. Five additional A2A-Cre mice received AVV5-DIO-eYFP virus. We tracked licking behavior in head-fixed mice performing an interval timing task (Toda et al., 2017). Mice received a 5 μL drop of 10% sucrose solution every 10 seconds (Figure 1D). Well-trained mice reliably generated anticipatory licking behavior before reward delivery (Figure 1E). Voluntary licking behavior in all mice consisted bouts of rhythmic protrusion and retractions of the tongue at a relatively fixed rate (4-8 Hz). All mice showed anticipatory licking starting 4-6s before the reward and peaking at roughly the expected time of reward delivery (Figure 1F).

94 We activated the direct pathway in the VLS in well-trained D1-Cre mice at three different
95 time points during the interval. We stimulated the direct pathway for 1 second before mice
96 normally initiate anticipatory licking, starting at 5 seconds before reward delivery (Figure 2A).
97 Though licking was generally sparse at this time point (Figure 2B, top), activation of direct
98 pathway MSNs resulted in large increases in licking rate (Figure 2B, bottom and Figure 2C,
99 Video S1). We determined the change in lick rate that resulted from laser stimulation by
100 calculating the lick rate obtained during the 1 second laser stimulation period for D1-Cre mice
101 and a control group that received eYFP virus. The normal lick rate obtained for the same time
102 point during trials without laser stimulation was subtracted from this value to quantify the laser-
103 induced change in lick rate (Figure 2D). A two-way ANOVA revealed a significant change in
104 licking rate by VLS direct pathway stimulation ($F = 56.4$, $p < 0.0001$) in addition to a significant
105 interaction between experimental group and laser frequency ($F = 8.76$, $p < 0.01$). We next
106 stimulated the direct pathway at 1 second prior to reward delivery (Figure 2E), during the time of
107 maximal anticipatory licking (Figure 2F, top). Although there was a ceiling effect on licking at
108 this time point (Figure 2F, bottom), we regularly observed a potentiation of the licking rate
109 during stimulation when compared to trials without stimulation (Figures 2G). A two-way
110 ANOVA revealed significant increase in licking rate as a result of VLS stimulation (Figure 2H,
111 $F = 54.11$, $p < 0.0001$), but no interaction between group and stimulation frequency ($F = 5.94$, p
112 $= 0.16$). In contrast to these effects, stimulation at the time of reward delivery (Figure 2I) had the
113 most modest impact on licking rate (Figures 2J and 2K). A two-way ANOVA detected a
114 significant effect of stimulation on licking even during consumption (Figure 2L, $F = 16.43$, $p <$
115 0.01), but did not show a significant interaction between group and stimulation frequency ($F =$
116 9.88, $p = 0.18$). Together, these results show for the first time that direct pathway stimulation in
117 the orofacial striatum can result in the generation of licking behavior. The effects of direct
118 pathway stimulation were less apparent when we stimulated during on-going self-generated
119 licking, which can be explained by a ceiling effect.
120 Remarkably, we observed that it was possible for licks to become entrained or time-
121 locked to laser pulses. We analyzed licking patterns in the frequency domain. Licking during
122 laser presentation could be shifted rightward toward a frequency of 10 Hz (Figure 3A, Video
123 S2). This appeared to be the upper limit of the central pattern generator for licking while, as
124 naturally generated licking showed a predominant frequency of 5-9 Hz (Figure 3B). We

quantified power spectral density (PSD) distributions for licking and determined the licking frequency that corresponded to the peak of the PSD during laser delivery and during naturally generated licking without stimulation (Figure 3C). Mice displayed two distinct licking frequency patterns, depending on whether licking occurred prior to or following reward. Laser stimulation further increased the licking frequency to around 10 Hz, which is rarely reached naturally. Indeed, we found that the peak power of licking was potentiated: there was an interaction between the effects of stimulation frequency and licking pattern on the peak of the PSD function (Figure 3D, two-way, mixed ANOVA, $F = 12.92$, $p < 0.05$). While we found a significant effect of stimulation on the actual licking frequency (Figure 3E, two-way mixed ANOVA, $F = 31.62$, $p < 0.0001$), there was no interaction between frequency and the pattern of licking ($F = 4.82$, $p = 0.23$). Stimulation also impacted peak PSD power in a similar manner when we stimulated the direct pathway in the middle of the interval at 5 seconds. We found a significant interaction between the effects of stimulation frequency and licking pattern on peak power (Figure 3F, $F = 13.28$, $p < 0.01$). We also observed a significant effect of stimulation on licking frequency (Figure 3G, $F = 43.22$, $p < 0.0001$), but no significant interaction between stimulation frequency and lick pattern ($F = 5.34$, $p = 0.1$). An interaction between the effects of licking type and stimulation frequency on peak power was even observed when stimulation occurred during consumption licking (Figure 3H, $F = 9.76$, $p < 0.05$), in addition to an increase in the licking frequency (Figure 5I, $F = 62.35$, $p < 0.001$). Interestingly, 5 Hz stimulation did not reduce the peak licking frequency. Regardless of stimulation frequency, light delivery to the VLS resulted in a positive shift in the frequency domain, above the naturally occurring states that mice normally generate. In addition, direct pathway stimulation could increase the consistency of licking at a given frequency, reflected in the PSD function, suggesting a role for the basal ganglia in controlling the duty cycle of the licking oscillator.

When stimulating the direct pathway at 5 seconds prior to reward, we observed that licking resulting from stimulation only began after a certain delay (Figure 4A), and that this delay was reduced with higher stimulation frequencies (Figure 4B). A one-way ANOVA revealed a significant effect of stimulation frequency on the latency to initiate licking following the onset of laser stimulation (Figure 4C; $F = 4.92$, $p < 0.01$). The lick bout that resulted from stimulation also lasted longer than the 1 second stimulation period. We counted the number of licks that occurred over the course of one second following the offset of stimulation and

compared that value with the number of licks normally detected during the same period in non-stimulation trials. Stimulation would result in some additional licking even after offset of stimulation, but this was not related to the stimulation frequency (Figure 4D; two-way mixed ANOVA; main effect of stimulation, $F = 12.23$, $p < 0.001$). These results show that greater stimulation of the direct pathway reduces licking initiation latency, and that licking continues for a short time beyond the immediate period of laser stimulation.

The indirect pathway primarily innervates the external globus pallidus, which then sends inhibitory projections to the basal ganglia output neurons in SNR. Activation of the indirect pathway therefore would disinhibit basal ganglia output. We stimulated MSNs expressing ChR2 driven by the A2A promoter in the VLS while mice performed the timing task. We found that in contrast to D1 activation, indirect pathway activation resulted in a pronounced suppression of licking. When stimulation occurred 5 seconds prior to reward delivery (Figure 5A), we observed a reduction in licking rate (Figures 5B and 5C). We found a significant effect of experimental group on the reduction in licking rate (Figure 5D, two-way ANOVA, $F = 18.56$, $p < 0.01$), but no interaction between experimental group and frequency ($F = 9.93$, $p = 0.11$). Although we did regularly observe a suppression of licking activity, the likelihood of licking at this period in the interval was low, reflecting a potential floor effect. We tested whether indirect pathway activation would as effectively suppress more vigorous licking behavior that is seen prior to reward. When activating the indirect pathway 1 second prior to reward (Figure 5E), we also observed a significant impact on anticipatory licking by indirect pathway stimulation (Figures 5F and 5G, Video S3). In addition to a strong suppression of licking by indirect pathway stimulation (Figure 5H, two-way ANOVA; significant effect of experimental group $F = 25.93$, $p < 0.0001$), we observed a significant interaction between the experimental group and stimulation frequency ($F = 13.83$, $p < 0.01$). We also tested the effect of indirect pathway activation on consumption licking that occurs after reward delivery (Figure 5I). Interestingly, we were less able to suppress licking that occurred following reward (Figures 5J and 5K). Nevertheless there was a significant effect of experimental group on the change in lick rate (Figure 5L, two-way ANOVA, $F = 8.37$, $p < 0.05$), in addition to a significant interaction between indirect pathway activation and licking suppression in the period following reward delivery ($F = 13.08$, $p < 0.05$). Together, these results reveal a frequency-dependent effect of indirect pathway stimulation on licking behavior, which can impact both anticipatory and consumption licking.

When we activated the indirect pathway at 5 seconds prior to reward delivery, when mice are least likely to lick, we observed a striking rebound of licking that followed the offset of stimulation (Figures 6A and B, Video S4). Rebound licking was characterized by higher licking rates during the second following laser offset as compared to the same time point in the absence of stimulation (Figure 6C, two-way mixed ANOVA, $F = 11.73$, $p < 0.01$), but there was no interaction between stimulation frequency and the rebound licking rate ($F = 1.16$, $p = 0.78$). We also quantified the latency to lick initiation following laser termination. Indirect pathway stimulation resulted in a reduced latency to initial lick onset following stimulation offset when compared to non-stimulation trials (Figure 6D, two-way mixed ANOVA, $F = 16.05$, $p < 0.001$). There was a significant interaction between trial type and the stimulation frequency, with higher frequencies resulting in lower onset latencies ($F = 16.82$, $p < 0.01$). Rebound lick onset latency also showed reduced variance, with higher stimulation frequencies resulting in more consistent rebound latencies (Figure 6E, two-way mixed 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 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 dependent on how many trials the mice received (Figure 7B). At higher frequencies, this gradual reduction of laser-generated licking rate was not as evident (Figure 7C). 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 onset latency (Figure 7E, $F = 3.03$, $p < 0.05$) 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 rebound licking increased as the session progressed, but this was most evident at higher stimulation frequencies (Figure 7G, two-way mixed ANOVA, interaction between trial quartile and stimulation frequency $F = 8.21$, $p < 0.05$, main effect of trial quartile F

218 = 5.12, $p < 0.05$). These results suggest that engagement of lower-level licking generating areas
219 by the basal ganglia can be modulated by motivational signals, but that this impact can be
220 overcome by higher direct pathway activity.

221 The basal ganglia have been extensively linked to timing, but investigation of the roles of
222 the direct and indirect pathway in mediating timing have not been explored. Our task allowed for
223 the interrogation of the roles of these pathways in timing, as mice will readily anticipate the
224 arrival of the reward (Figure 8A). We incorporated probe trials into the behavioral sessions in
225 which the delivery of a reward was not followed by the expected reward 10 seconds later (Figure
226 8B). The probe trials are not cued.. We found that all mice generated a discrete bout of licking
227 that reflects the operation of an internal timing process, with a peak that accurately reflects the
228 expected time of reward delivery (Roberts, 1981). Prior work manipulating neural function of the
229 basal ganglia has revealed alterations in the peak during probe trials (Meck, 2006). On a fraction
230 of the probe trials we performed stimulation of the direct and indirect pathways to examine their
231 contributions to timing.

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

245 We also tested the effect of indirect pathway stimulation on timing during probe trials. As
246 observed previously, stimulation of the indirect pathway at 5 seconds after reward resulted in a
247 rebound in licking following the termination of stimulation. This was also the case during peak
248 probe trials. Mice showed two different 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 a bout of licking ('recovery' pattern, Figure 10A). In addition, rebound licking could also initiate the peak, so that the peak lasts longer than usual ('absorb' pattern, Figure 10B). Sometimes, mice also generate a peak without showing a clear rebound following stimulation ('no-rebound' pattern, Figure 10C). These different patterns (recovery, absorb, and no-rebound) were equally common (Figure 10D, n = 5, one-way RM ANOVA; $F = 0.2$, $p > 0.05$). Interestingly, peak analysis on these three licking patterns revealed distinct effects of indirect pathway stimulation on interval timing. For each pattern of licking, we compared the mean peak time with the peak time on trials in the same session that did not contain laser stimulation. During recovery-pattern 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 increased duration (Figure 10F, two-tailed t-test, $p < 0.05$).

Finally, we asked if the behavior following indirect pathway stimulation was altered as the session progressed. We found that probability of detecting a recovery pattern decreased as the session progressed, whereas the likelihood of detecting no-rebound patterns 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' pattern.

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 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), 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).

Thus our results shed light on the interactions of the direct and indirect pathways in top-down regulation of 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.

Given the high temporal resolution of our behavioral measures and the highly repetitive nature of the licking behavior, we were able to analyze licking in the frequency domain. We showed for the first time that top-down modulation of central patterns generators takes the form

311 of duty cycle regulation. That is to say, the descending signals from the basal ganglia modulate
312 the 'on time' of a relatively stereotyped pattern generator, presumably in the brainstem.
313 Interestingly, these effects saturate above 25 Hz for stimulation of either pathway. That is, the
314 strongest possible duty cycle modulation (proportion of on time or off for the lick pattern
315 generator) is achieved at this stimulation frequency. We found that licking frequency could enter
316 a high-frequency mode that exceeded the frequency ranges normally observed in mice, but the
317 highest possible rate of licking is still around 9-10 Hz, which appears to be the limit of the lick
318 pattern generator. Normally, the lick pattern generator oscillates at 5-6 Hz for anticipatory
319 licking and 6-8 Hz for consummatory licking. When stimulation frequency exceeded 10 Hz, the
320 licking frequency can no longer increase further. Rather the probability of licking at 9-10 Hz
321 increased. Stronger signals from the direct pathway can increase the lick frequency slightly, but
322 the major effect is to increase the proportion of time that the pattern generator is turned on (duty
323 cycle). In some cases, with 10 Hz stimulation we could synchronize the lick cycle such that each
324 light pulse would be followed by a single lick (**Figure 3**). This higher-frequency mode was not
325 sensitive to laser stimulation frequency, suggesting the CPG operates in several discrete
326 frequency settings, rather than being able to modulate their output in a continuous manner (Rossi
327 et al. 2016). This is analogous to a fan with discrete settings, such as off, low, and high, and also
328 similar to the operation of other brain stem systems, such as those regulating locomotor pattern
329 generators (Bellardita & Kiehn, 2015).
330 We observed a reliable rebound of licking following indirect pathway stimulation in the
331 middle of the interval (**Figure 6**). This was only possible to detect because mice rarely lick at
332 that time point in our task, and the effect was not masked by the delivery of reward or the
333 presence of consumption licking. In addition to the increase in licking rate, we also observed an
334 effect of stimulation frequency on latency to rebound licking. Indirect pathway stimulation may
335 result in more active nigral output, which results in a large suppression of downstream structures.
336 The release of this inhibition then gives rise to a burst of rebound activity in regions that are
337 responsible for lick generation, resulting in fast reactivation of licking CPGs above the threshold,
338 and a sudden output of behavior at a consistent latency.
339 The effects of direct and indirect pathway stimulation were sensitive to a motivational
340 variable, as the latencies to licking, overall licking rates, and the latency to rebound were
341 observed to alter over the course of the session (**Figure 7**). These results can also be explained

342 by the possibility that, over time, the CPG threshold that must be exceeded to drive licking can
343 be increased, or that the activating signal attenuates as mice become sated.
344 We previously proposed that different classes of SNr projection neurons operate in
345 opposition during the deviation of movements away from neutral postures or states (Barter et al.,
346 2015; Fan, Rossi, & Yin, 2012; Yin, 2014). The present results suggest a potential role of the
347 direct and indirect pathways for modulating similar opponent output channels for the orofacial
348 system. The direct pathway is sufficient for bringing the activity of lick-generating centers above
349 a threshold; however, the indirect pathway may play an important role in maintaining output
350 within a specific range once above that threshold. We could expose the physical limits of the
351 system by selectively driving the direct pathway, though mice rarely enter this regime on their
352 own. Because of the concurrent operation of the indirect pathway, output is rarely expressed at
353 its maximum level. It is only under conditions of artificially targeting these two pathways
354 separately that behavior can be described as operating in an all-or-none, go/no-go fashion. Prior
355 observations of coincident activity of the two pathways may reflect coordinated regulation of
356 behavior to keep output within a certain range. The balanced relationship between these two
357 pathways may be responsible for the continuous adjustments to specific kinematic details of
358 ongoing movement.
359 Our results also shed light on the distinct contributions of direct and indirect pathways to
360 timing. The basal ganglia have long been implicated in interval timing (Meck, 2006). However,
361 little has been done to explore the relative contributions of the direct and indirect pathways in
362 interval timing (Frederick & Allen, 1996). The use of the fixed-interval schedule allowed us to
363 incorporate peak probe trials that measure timing of goal-directed behavior.
364 We found that stimulation of the direct pathway reset the interval timing mechanism and
365 activation of the indirect pathway could pause its operation (**Figures 9 & 10**). These
366 observations are in accord with previous pacemaker-accumulator models (Gibbon et al., 1984).
367 During peak probe trials, mice show an average rate of responding that peaks at the trained
368 interval of 10 seconds. When stimulating the direct pathway, we found that the peak would
369 appear another 10 seconds following the onset of stimulation. The interval was maintained, but
370 the activation of the direct pathway appeared to reinitiate the accumulation of the 10 second
371 interval, like resetting some 'internal clock'.
372 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 excessive direct pathway activity. We found a complex interaction between the timing system and the rebound licking immediately following 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 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 accumulation on a given trial would allow the rebound licking to initiate the peak, whereas with slower accumulation the indirect pathway activity could only interrupt or pause this process. In partial support of this idea are our observations of the motivational effects on behavioral responses to indirect pathway stimulation.. As rebound licking latency increases over time, the proportion of peak-probe trials that contained rebound licking also decreased.

In summary, we show that direct pathway stimulation in the ventrolateral striatum can initiate licking, whereas indirect pathway stimulation can suppress ongoing licking, and that these pathways can work in concert to continuously regulate ongoing licking. In addition, the distinct effects of direct and indirect stimulation on timing behavior suggest that each pathway differentially interacts with the internal time-keeping system: direct pathway stimulation can reset the timer, whereas indirect pathway activation can only pause or interfere it. These principles may be extended to explaining how the basal ganglia regulate volitional actions in general.

Methods

404

405 *Subjects:* 8 adult D1-Cre and 13 A2A-Cre mice of both sexes were used (Jackson Labs, Bar
406 Harbor, ME). These mice expressed Cre-recombinase only in D1 or A2A striatal neurons,
407 respectively. All experiments were conducted during the light phase of the 12:12 light cycle.
408 Mice had unrestricted access to food in their home cages, but were water deprived and received a
409 10% sucrose solution during behavioral sessions. Body weight was maintained at 85% body
410 weight and was monitored on a daily basis, with additional water given after experimental
411 training as needed. All experimental procedures were approved by the Duke University
412 Institutional Animal Care and Use Committee.
413
414 *Viral vectors*: AAV5-EF1α-DIO-hChR2(H134R)-eYFP was injected into 8 D1-Cre and 8 A2A-
415 Cre mice. AAV5-EF1α-DIO-eYFP was injected into 5 A2A-Cre mice for control experiments.
416 Vectors were obtained from the University of North Carolina Vector Core.
417
418 *Surgery*: Mice were placed in a stereotaxic frame and anesthetized with ~1-1.5% isoflurane.
419 Small holes were drilled bilaterally at AP: +0.2 mm and ML: +/- 2.5 mm relative to bregma.
420 Stereotaxically guided injections of (200 nL per hemisphere) were made at AP: +0.2 and ML:
421 2.5 and DV: -4.6 & -4.4 relative to bregma using a Nanoject III microinjector (Drummond
422 Scientific, Broomall, PA). 100 µm-core optic fibers (Precision Fiber Products, Chula Vista, CA)
423 were implanted with the same AP and ML coordinates and at DV: -4.2 in order to target cell
424 bodies of the VLS. Fibers were secured to the skull with screws and dental cement along with a
425 head post for head fixation.
426
427 *System for behavioral training and recording*: Mice were allowed 7-14 days to recover from
428 surgery and then were water deprived in their home cages. The interval timing task is the same as
429 previously described (Toda et al., 2017). Briefly, behavioral experiments were conducted in a
430 sound-proof chamber with the mice perched in a custom-made elevated tunnel platform. For
431 head fixation, an implanted steel head post was clamped on both sides of the head. A metal
432 drinking spout was positioned directly in front of the mouth so that the mouse only needs to
433 protrude its tongue to access the spout. A 10% sucrose solution was gravity fed to the spout and
434 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 mice 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, mice 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 mice showed anticipatory licking behavior.

Peak probe trials: Once mice 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 quantify the internal representation of time. 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. Mice 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.

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Figure 1

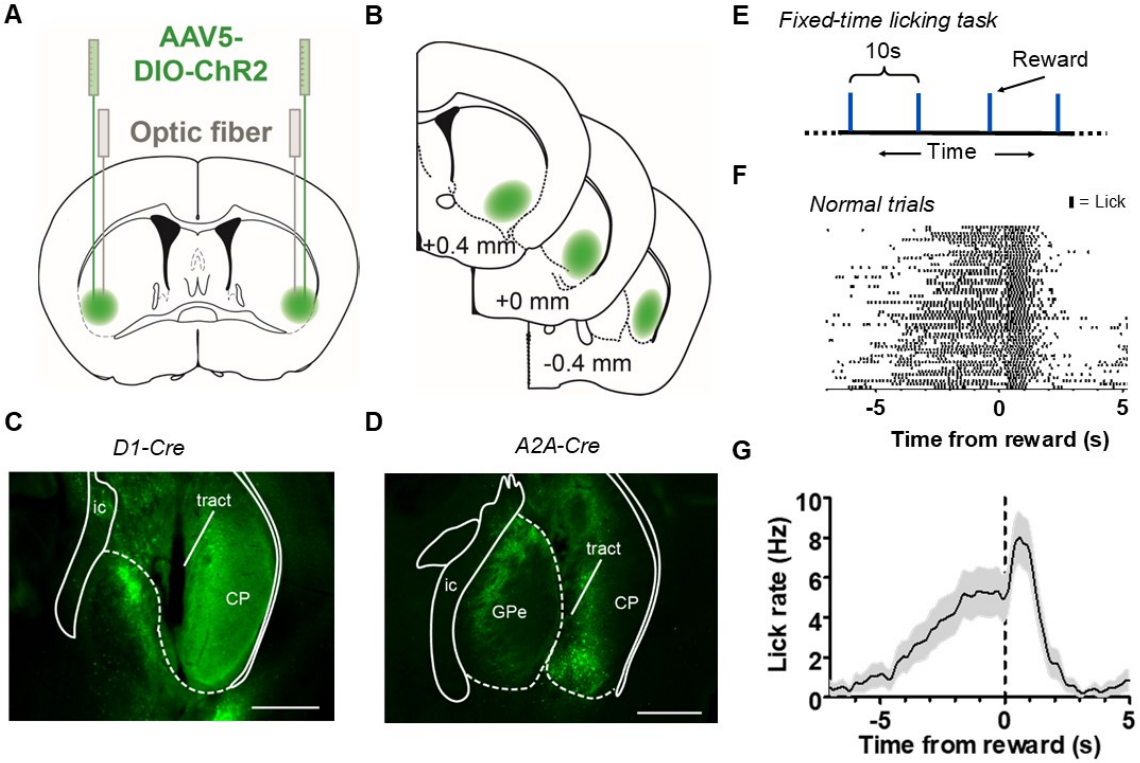
A
AAV5-
DIO-ChR2
Optic fiber
B
+0.4 mm
+0 mm
-0.4 mm
C
D1-Cre
ic
tract
CP
D
A2A-Cre
ic
GPe
tract
CP
E
Fixed-time licking task
10s
Reward
Time
F
Normal trials
= Lick
-5
0
5
Time from reward (s)
G
Lick rate (Hz)
10
8
6
4
2
0
-5
0
5
Time from reward (s)

Figure 2

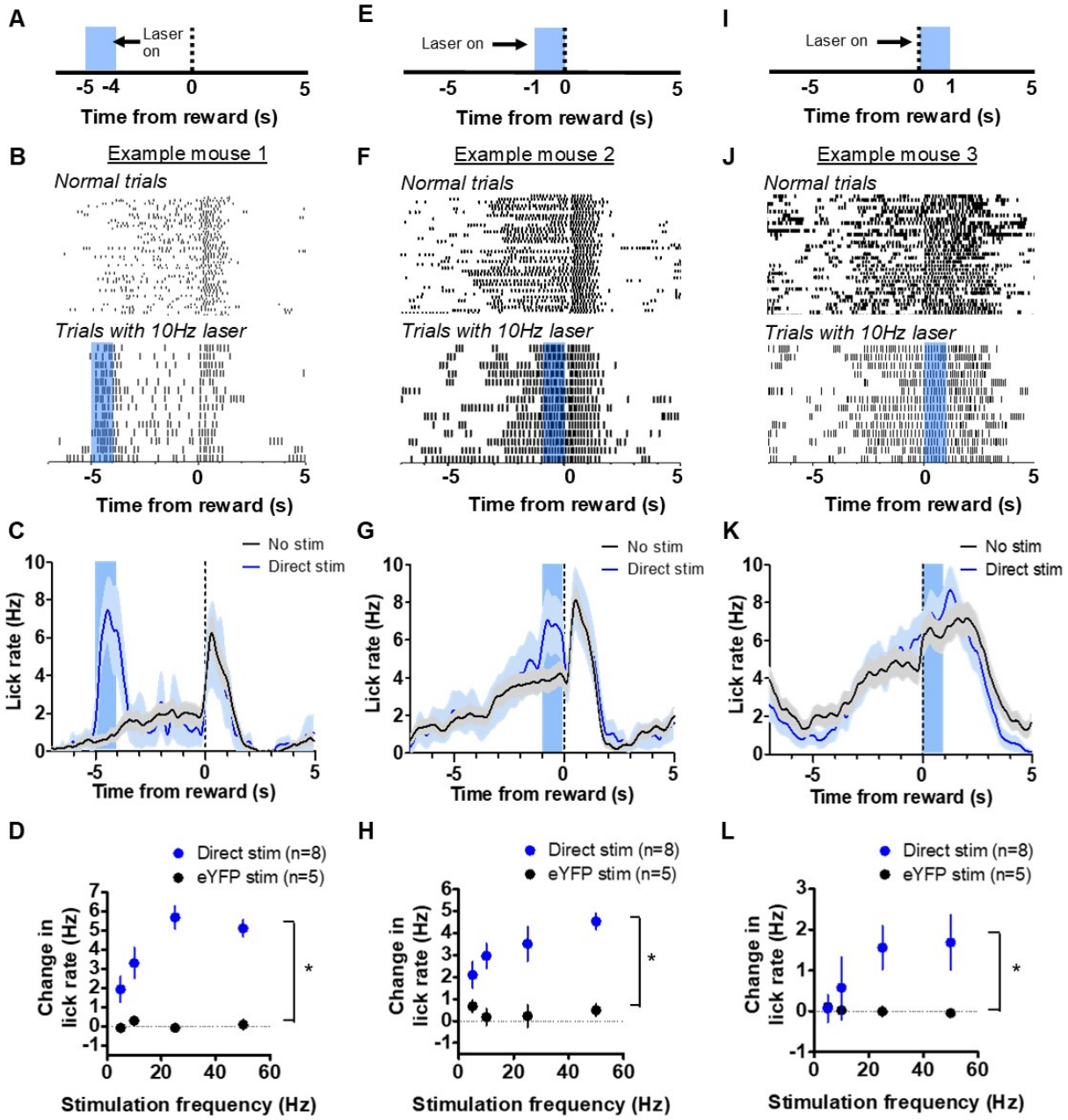
A
Laser on
-5 -4 0 5
Time from reward (s)
E
Laser on
-5 -1 0 5
Time from reward (s)
I
Laser on
-5 0 1 5
Time from reward (s)
B
Example mouse 1
Normal trials
Trials with 10Hz laser
-5 0 5
Time from reward (s)
F
Example mouse 2
Normal trials
Trials with 10Hz laser
-5 0 5
Time from reward (s)
J
Example 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

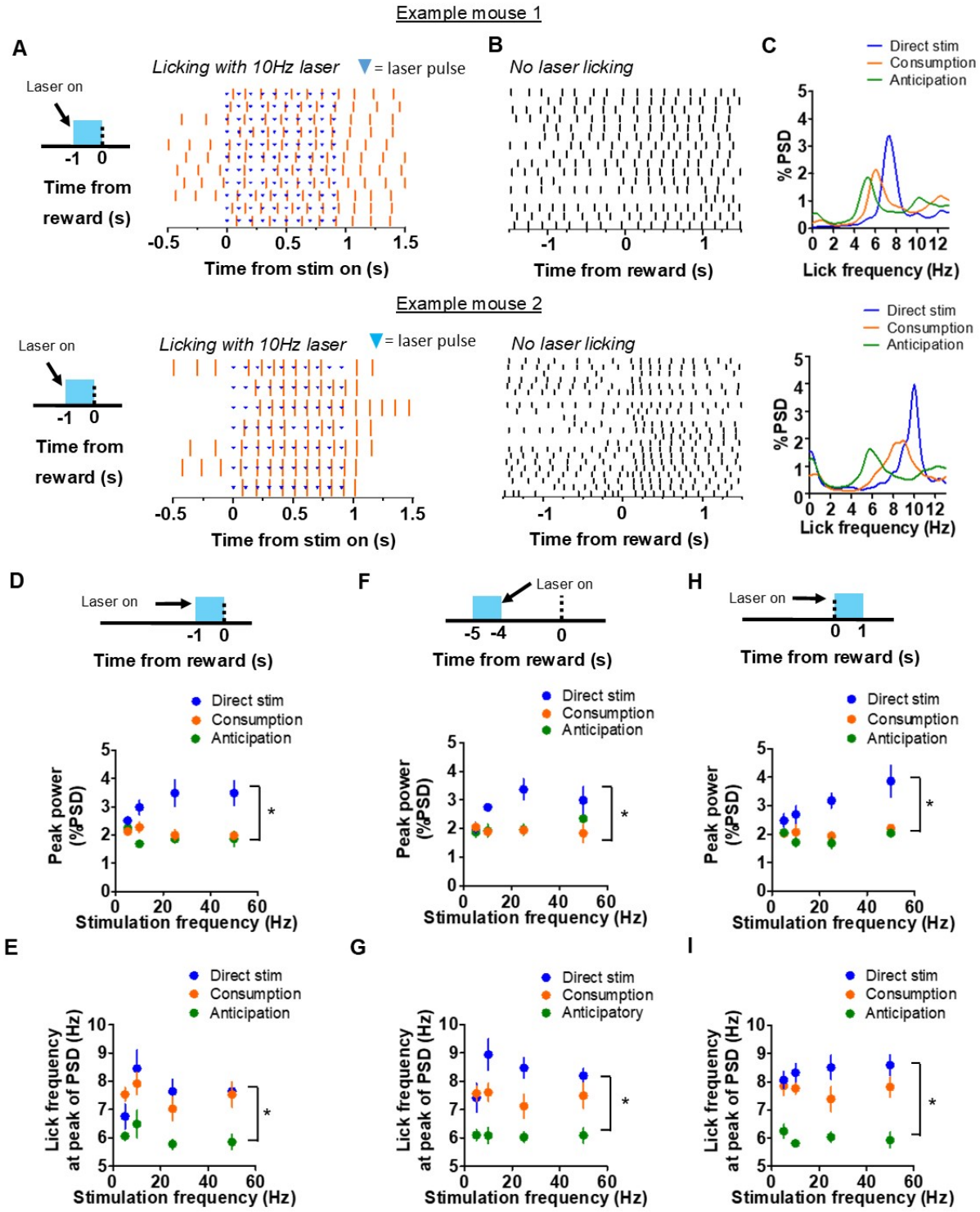
Example mouse 1
A
Laser on
-1 0
Time from reward (s)
Licking with 10Hz laser
= laser pulse
-0.5 0 0.5 1 1.5
Time from stim on (s)
B
No laser licking
-1 0 1
Time from reward (s)
C
Direct stim
Consumption
Anticipation
% PSD
0 2 4 6 8 10 12
Lick frequency (Hz)
Example mouse 2
Laser on
-1 0
Time from reward (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

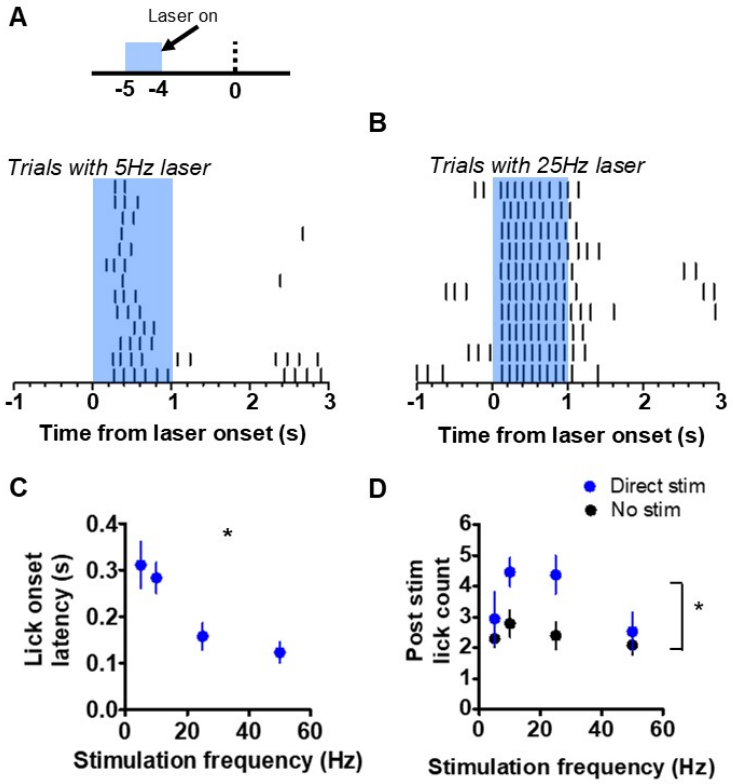
A
Laser on
-5 -4
0
B
Trials with 5Hz laser
Trials with 25Hz laser
-1 0 1 2 3
Time from laser onset (s)
C
D
Direct stim
No stim
Lick onset latency (s)
0.0 0.1 0.2 0.3 0.4
0 20 40 60
Stimulation frequency (Hz)
Post stim lick count
0 1 2 3 4 5 6
*

Figure 5

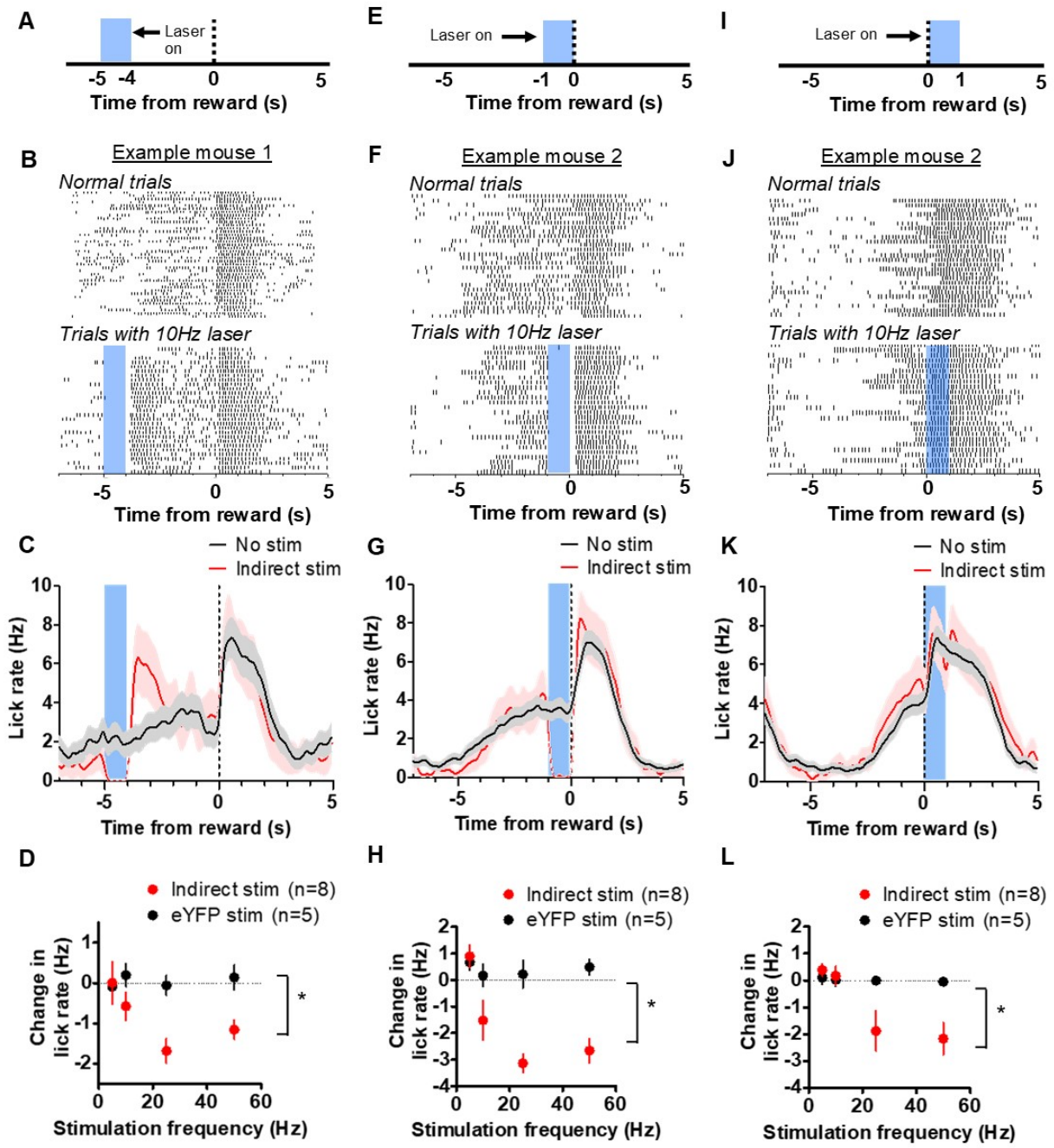
A
Laser on
-5 -4 0 5
Time from reward (s)
E
Laser on
-5 -1 0 5
Time from reward (s)
I
Laser on
-5 0 1 5
Time from reward (s)
B
Example mouse 1
Normal trials
Trials with 10Hz laser
Time from reward (s)
F
Example mouse 2
Normal trials
Trials with 10Hz laser
Time from reward (s)
J
Example mouse 2
Normal trials
Trials with 10Hz laser
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

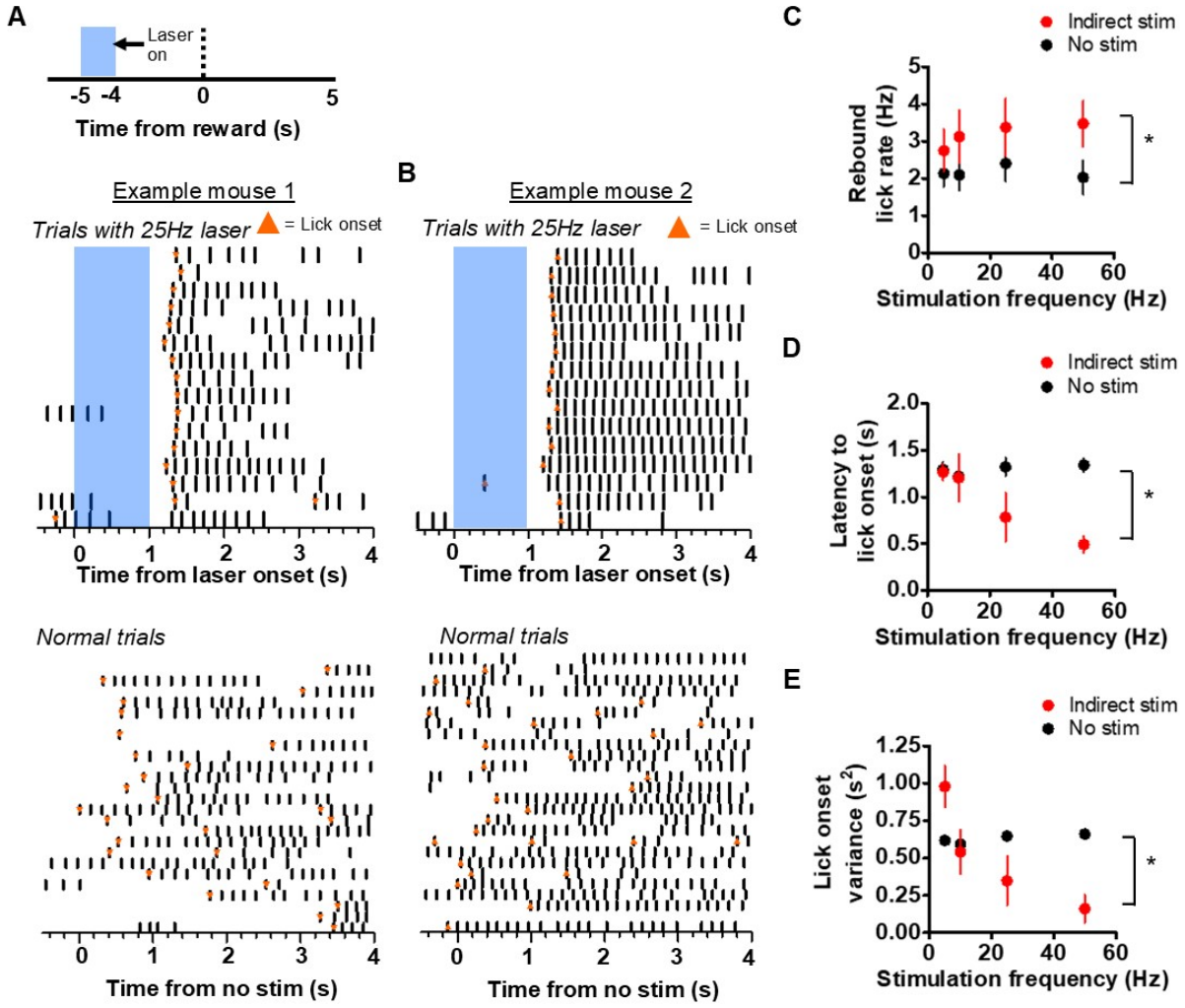
A
Laser on
-5 -4 0 5
Time from reward (s)
B
Example mouse 1
Trials with 25Hz laser
= Lick onset
Time from laser onset (s)
Normal trials
Time from no stim (s)
Example mouse 2
Trials with 25Hz laser
= Lick onset
Time from laser onset (s)
Normal trials
Time from no stim (s)
C
Indirect stim
No stim
Rebound 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

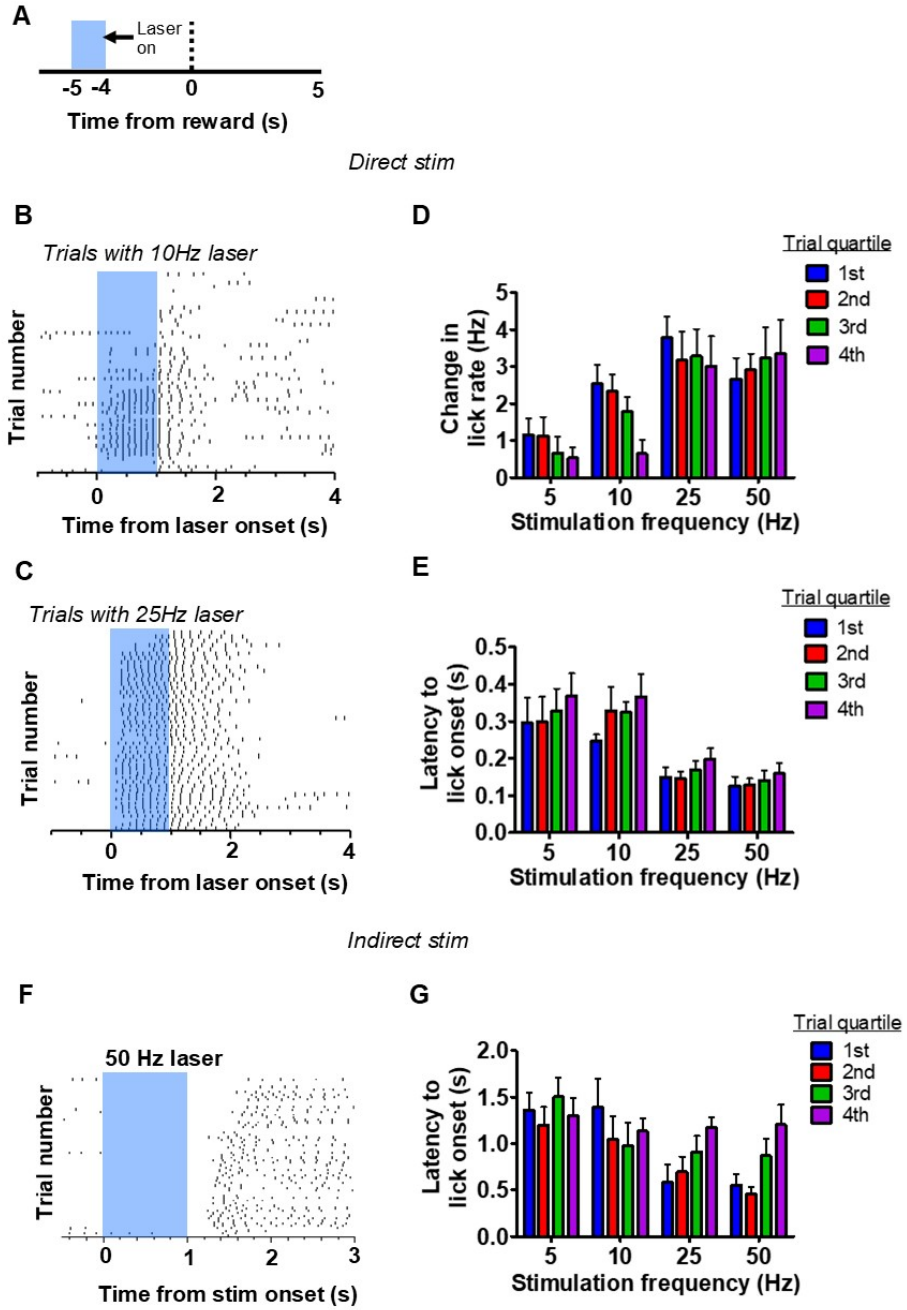
A
Laser on
-5 -4 0 5
Time from reward (s)
Direct stim
B
Trials with 10Hz laser
Trial number
0 2 4
Time from laser onset (s)
C
Trials with 25Hz laser
Trial number
0 2 4
Time from laser onset (s)
D
Trial quartile
1st
2nd
3rd
4th
Change in lick rate (Hz)
0 1 2 3 4 5
5 10 25 50
Stimulation frequency (Hz)
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

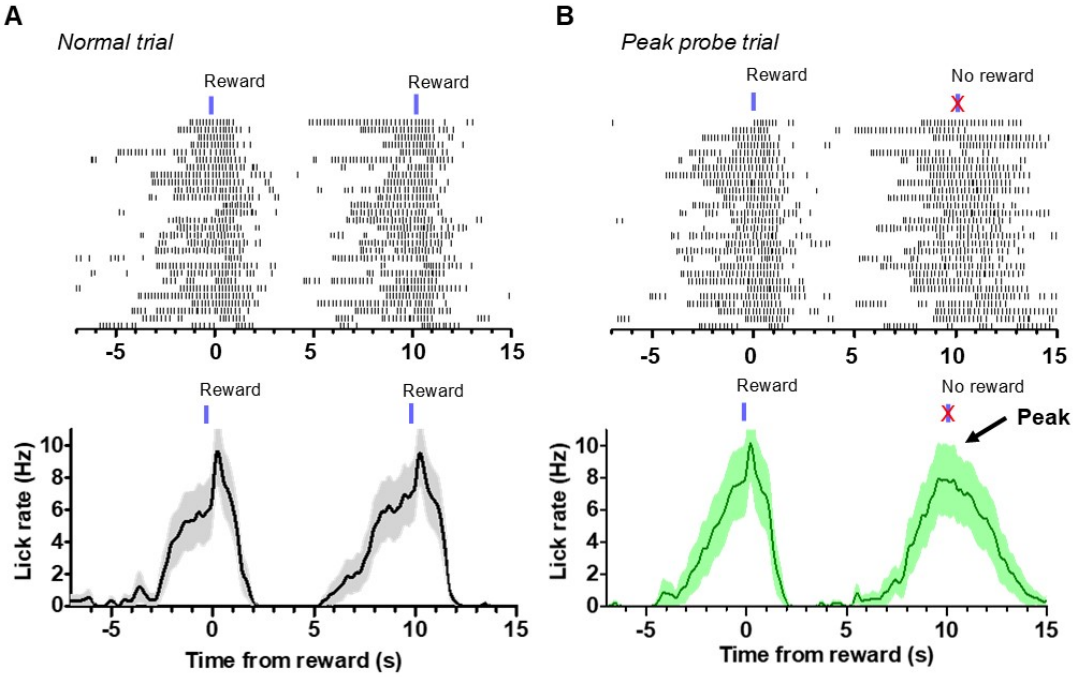
A
Normal trial
Reward
Reward
-5
0
5
10
15
Reward
Reward
Lick rate (Hz)
10
8
6
4
2
0
-5
0
5
10
15
Time from reward (s)
B
Peak probe trial
Reward
No reward
-5
0
5
10
15
Reward
No reward
Peak
Lick rate (Hz)
10
8
6
4
2
0
-5
0
5
10
15
Time from reward (s)

Figure 9

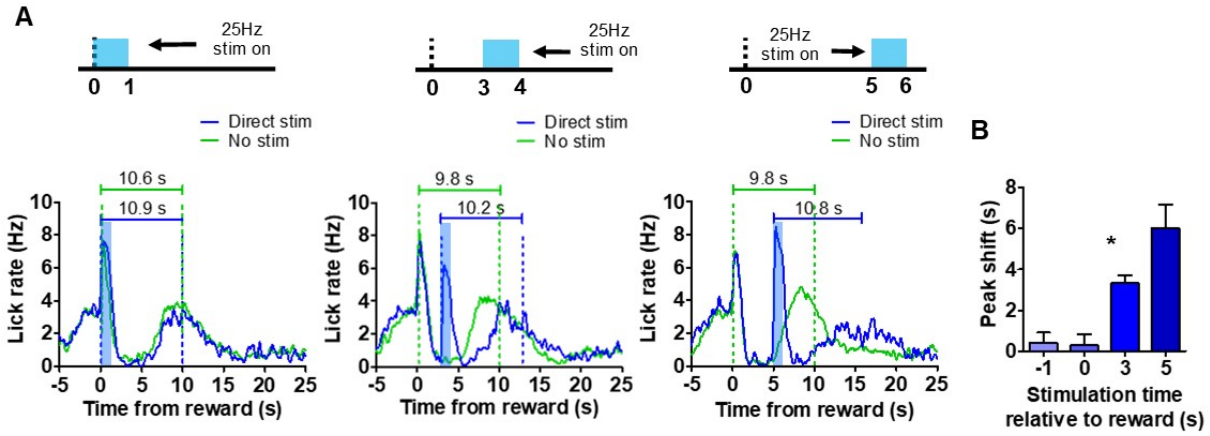
A
25Hz
stim on
0 1
0 3 4
0 5 6
Direct stim
No stim
10.6 s
10.9 s
9.8 s
10.2 s
9.8 s
10.8 s
Lick rate (Hz)
Time from reward (s)
B
Peak shift (s)
*
-1 0 3 5
Stimulation time
relative to reward (s)

Figure 10

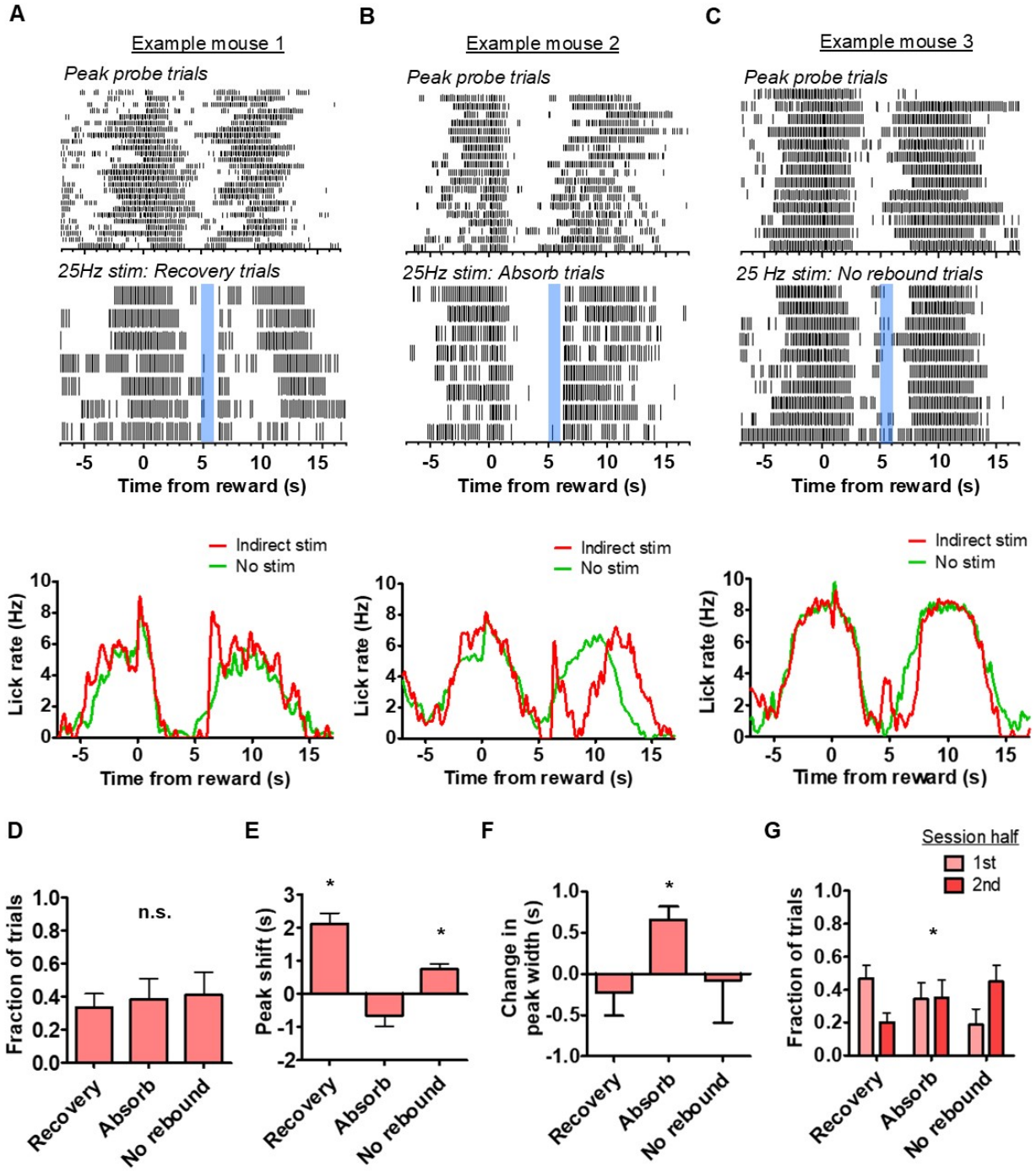
A
Example mouse 1
Peak probe trials
25Hz stim: Recovery trials
B
Example mouse 2
Peak probe trials
25Hz stim: Absorb trials
C
Example mouse 3
Peak probe trials
25 Hz stim: No rebound trials
Time from reward (s)
Indirect stim
No stim
Lick rate (Hz)
D
Fraction of trials
n.s.
Recovery
Absorb
No rebound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

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 mice. Mice receive a 10% sucrose reward (5 µL) every 10 seconds. **F**) Example lick raster of a well-trained mouse 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 from the same mouse 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 experimental group: $F = 56.4$, $p < 0.0001$; 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 from the same mouse 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 mice 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 normal 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 immediately 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 pattern, $F = 48.01$, $p < 0.0001$; interaction Lick pattern 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 (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 (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: $F = 12.23$, $p < 0.001$; interaction Trial type vs. Frequency, $F = 3.88$, $p = 0.15$). Error bars denote SEM.

Figure 5. Stimulation of the indirect pathway in the orofacial striatum suppresses 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 from the same mouse 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 Experimental Group, $F = 18.56$, $p < 0.01$). **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 show licking from the same mouse 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 Group, $F = 25.93$, $p < 0.0001$; interaction Frequency vs Group, $F = 13.83$, $p < 0.01$). **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 mouse 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 Group, $F = 8.37$, $p < 0.05$; interaction Group vs Frequency, $F = 13.08$, $p < 0.05$). 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 no-stimulation trials from the same mouse, 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 another mouse showing rebound licking after indirect pathway stimulation. *Bottom*: Raster plot of licking during non-stimulation trials from the same mouse 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: $F = 16.05$, $p < 0.001$; interaction Stimulation vs 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, $F = 20.66$, $p < 0.01$; Frequency 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 mouse but a 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 Quartile, $F = 1.69$, $p < 0.05$; effect of Frequency, $F = 31.13$, $p < 0.01$; interaction Quartile vs 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 (two-way mixed ANOVA, effect of Quartile, $F = 3.03$, $p < 0.05$; effect of Frequency, $F = 35.34$, $p < 0.01$; interaction Quartile vs 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 Quartile, $F = 5.21$, $p < 0.05$; effect of Frequency, $F = 18.18$, $p < 0.05$; interaction Quartile vs 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 mouse 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 bars reflect the population mean peak times 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 bars reflect the population mean peak times 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 bars reflect the population mean peak times 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 and a recovery in peak licking. Both rasters reflect licking from the same mouse in the same session. *Bottom*: Mean lick rate calculated from the example session shown above for peak probe trials without laser stimulation and stimulation trials showing a "recovery" pattern. **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 show licking from the same mouse in the same session. *Bottom*: Mean lick rate calculated from the example session shown above for peak probe trials without laser stimulation and stimulation trials showing an "absorb" pattern. **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. *Bottom*: Mean lick rate calculated from the example session shown above for peak probe trials without laser stimulation and stimulation trials showing a "no rebound" pattern. **D**) The three types of patterns 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 pattern by the mean peak time without stimulation. 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 showing each pattern during the first and second halves of the behavioral session. On the whole, the recovery pattern was gradually replaced with no rebound pattern (two-way, mixed ANOVA, interaction trial type x session half, $F = 25.61$, $p < 0.01$). Error bars denote SEM.