# Nucleus accumbens D2-expressing neurons: Balancing reward and licking disruption through rhythmic optogenetic stimulation

Nikte Requejo-Mendoza[1,2], José-Antonio Arias-Montaño[1], Ranier Gutierrez[2,3]*

[1] Departamento de Fisiología, Biofísica y Neurociencias, Centro de Investigación y de Estudios Avanzados (Cinvestav), Ciudad de México, México

[2]Laboratory Neurobiology of Appetite; Departamento Farmacología, Centro de Investigación y de Estudios Avanzados (Cinvestav), Ciudad de México, México

[3] Laboratory Neurobiology of Appetite; Centro de Investigación sobre el Envejecimiento, Centro de Investigación y de Estudios Avanzados (Cinvestav Sede sur), Ciudad de México, México.

*Corresponding author: Email: ranier@cinvestav.mx

# Abstract

Dopamine D1 receptor-expressing neurons in the nucleus accumbens (NAc) are known to be critical for processing reward and regulating food intake. However, the role of D2-expressing neurons in this region remains less understood. This study employed optogenetic manipulations to investigate the role of NAc D2-expressing neurons in reward processing and sucrose consumption. Optogenetic activation of these neurons decreased sucrose preference (at 20 Hz), disrupted licking patterns (particularly at 8 and 20 Hz), and increased self-stimulation. Conversely, synchronizing stimulation with the animal's licking rhythm mitigated licking disruption and even increased sucrose intake, suggesting a rewarding effect. Furthermore, 20 Hz stimulation (but not 8 Hz) induced place preference in a real-time place preference (RTPP) test, establishing a long-lasting spatial reward memory. In contrast, inhibiting D2 neurons produced a negative hedonic state, although not reaching a complete aversion, influencing food choices in specific contexts. For instance, while the RTPP test per se was not sensitive enough to observe place aversion when mice could choose between consuming a high-fat diet (HFD) pellet in a context associated with or without inhibition of D2 neurons, they preferred to consume HFD on the non-inhibited side. This suggests that the palatability of HFD can unmask (but also overshadow) the negative hedonic state associated with D2 neuron inhibition. A negative reinforcement paradigm further confirmed the active avoidance behavior induced by D2 neuron inhibition. In conclusion, NAc D2 neuron inhibition induces a negative hedonic state, while activation has a dual effect—it is rewarding yet disrupts licking behavior—highlighting its complex role in reward and consummatory behavior. Importantly, self-paced stimulation entrained to licking rhythms offers a more “physiologically” relevant approach to brain activation.

**Keywords:** Reward; Sucrose; Licking; Aversion; Nucleus Accumbens Shell; Medium Spiny Neurons.

# Introduction

The nucleus accumbens (NAc) is known to be involved in reward processing and food intake.[1–3] While the role of dopamine D1 receptor-expressing neurons in the NAc has been extensively studied, the role of D2 neurons remains unclear.[4,5] D1 medium spiny neurons (MSNs) in the NAc are well-established as key players in processing rewards and regulating food intake.[6,7] For instance, D1 neuron inhibition permits food consumption, whereas activation suppresses licking behavior, a fundamental component of the feeding-gating hypothesis of the NAc, particularly of the shell region.[8,9] In contrast, feeding has been reported to have no modulatory effect on D2 receptor-expressing neurons.[9,10] Accordingly, a recent study using rabies virus has traced that D1 NAc neurons receive direct inputs from taste bud cells on the tongue and send direct projections to the reticular formation in which the Central Pattern Generator (CPG) for rhythmic licking is controlled. In contrast, the same study showed that D2 neurons are not directly connected with the CPG controlling tongue movements.[11]

Beyond its involvement in food consumption, the NAc has been recognized as a crucial player in reward processing since the groundbreaking work of James Olds (1956) on the brain pleasure centers.[12] This role is also supported by evidence that mice will self-stimulate to activate all glutamatergic inputs to the NAc shell, [13,14] indicating the capability of glutamate to modulate reward and even suppress feeding behavior,

presumably by influencing the activity of either D1 or D2 receptor-expressing neurons.[14] The classic view of D1- and D2-expressing neurons posited opposing roles in feeding[15] and in reward, D1 neuron activation being rewarding and D2 activation aversive, with inhibition leading to the opposite effects.[16] However, current research suggests a more complex dynamic. While D1-expressing neurons predominantly drive positive reinforcement and reward behaviors,[16–18] D2-expressing neurons appear to have more complex, bidirectional effects, depending on the context and neural stimulation patterns.[4] Soares-Cunha work further challenged the classic view, demonstrating that brief (1 s, 40 Hz) optogenetic stimulation of D1- or D2-expressing neurons is rewarding, whereas prolonged stimulation (60 s) elicits aversion.[19] This highlights that the rewarding or aversive nature of D1 and D2 neuron activity hinges on stimulation parameters.[5] Thus, while the role of NAc D1-expressing neurons in reward is more established,[20,21] the contribution of D2-expressing neurons yields variable results across different behaviors and experimental setups.[4,5,11,17]

This study employed optogenetic manipulations to investigate the specific role of NAc D2-expressing neurons in reward processing and consummatory behavior related to sucrose and HFD intake. By exploring multiple frequency-dependent parameters for optogenetic manipulation, we aimed to provide further insights into how D2 neurons fine-tune reward processing and consummatory behaviors. Our data demonstrate the complex nature of NAc D2 neuron activation, revealing its dual role in promoting reward while disrupting certain aspects of consummatory behavior. In contrast, NAc D2 neuron inhibition induces a negative hedonic value. Furthermore, our findings highlight the importance of stimulation parameters in understanding NAc D2 neuron function

# Results

## Optogenetic activation of NAc D2-expressing neurons at 20 Hz reduces sucrose preference by disrupting the microstructure of licking and increasing self-stimulation

A two-bottle freely licking preference test was conducted to assess the role of NAc D2-expressing neurons in modulating sucrose preference. This experiment was conducted under three conditions, each lasting two days: 1) Baseline (No-laser), 2) Laser activated by the sucrose bottle, and 3) Laser activated by the water bottle. In the $D2^{tdTomato}$ (control) and $D2^{ArchT}$ (inhibition) groups, animals maintained a stable preference for sucrose over water throughout all conditions. However, in the $D2^{ChR2}$ (activation) group, optogenetic stimulation at 20 Hz during sucrose consumption significantly reduced sucrose preference (**Fig 1c** [* one-way ANOVA; main factor: groups (Laser sucrose condition); $F_{(2, 62)} = 9.20$, $p < 0.001$]), a *post hoc* analysis further revealed that $D2^{ChR2}$ exhibited a significant decrease compared to $D2^{tdTomato}$ and $D2^{ArchT}$ (all *p's < 0.003). This reduction in sucrose preference was accompanied by a marked increase in water preference, as indicated by fewer total sucrose licks and more water licks in the $D2^{ChR2}$ group (**Fig 1d** [* one-way ANOVA; main factor: groups (within Laser sucrose condition); $F_{(1, 40)} = 13.81$, $p < 0.001$], [# one-way ANOVA; main factor: conditions; for water $F_{(1, 44)} = 24.13$, $p < 0.001$; for sucrose $F_{(1, 44)} = 13.30$, $p < 0.001$]). As well as an increase in total licks (water+ sucrose) within the same condition for the $D2^{ArchT}$ group [@ one-way ANOVA; main factor: conditions; $F_{(1,39)} = 4.17$, $p = 0.04$]. Additionally, when stimulation was paired with the

water bottle, the $D2^{ChR2}$ group exhibited a significant increase in sucrose licking compared to the baseline condition [# one-way ANOVA; main factor: conditions; $F_{(1,36)} = 7.75$, $p = 0.008$], the $D2^{tdTomato}$ group [* one-way ANOVA; main factor: groups; $F_{(1,26)} = 27.91$, $p<0.001$] and generating a significant increase for total licks [@ one-way ANOVA; main factor: groups; $F_{(1,26)} = 31.66$, $p<0.001$], as well the $D2^{ArchT}$ group showed a significant increase compared with $D2^{tdTomato}$ group [* one-way ANOVA; main factor: groups; $F_{(1,21)} = 12.40$, $p = 0.002$], resulting in a significant increase for total licks [@ one-way ANOVA; main factor: groups; $F_{(1,21)} = 11.09$, $p = 0.003$]. This increase in licking behavior was accompanied by a higher number of trials initiated (a measure of self-stimulations) (**Fig 1e** [*one-way ANOVA; main factor: groups; $F_{(2, 62)} = 11.67$, $p < 0.001$]), suggesting a rewarding effect induced by 20 Hz stimulation. Additionally, comparisons between the laser and No-laser in Baseline condition in the $D2^{ChR2}$ group showed significant differences for sucrose licks (L_s) (paired t-test; $t_{(44)} = 2.72$, $p = 0.0092$) and water licks (L_w) (paired t-test; $t_{(36)} = 3.71$, $p = 0.0007$). A deeper analysis of the licking microstructure revealed that D2 neuron activation disrupted normal licking patterns (**Table 1**). Heatmaps of licking behavior (**Figs 1f-h**) revealed consistent licking patterns across all groups during baseline conditions (**Fig 1f**). However, optogenetic stimulation paired with sucrose consumption (**Fig 1g**) induced a significant decrease in lick bout durations, specifically within the $D2^{ChR2}$ group [one-way ANOVA; main factor: groups (within Laser sucrose condition) $F_{(1, 44)} = 35.90$, $p < 0.001$] and reduced bout size [one-way ANOVA $F_{(1, 44)} = 13.30$, $p < 0.001$], accompanied by increased number of licking bouts. A similar fragmentation in licking microstructure was observed when water was paired with stimulation (**Fig 1h**) for bout durations [one-way ANOVA; groups; $F_{(1, 36)}$ =

127 7.73, $p < 0.001$] and bout size [one-way ANOVA; groups; $F_{(1, 36)} = 10.10$, $p < 0.001$].
128 Further analysis of licking microstructural parameters (**Table 1**) confirmed that
129 optogenetic activation of D2 neurons significantly reduced bout size and duration while
130 increasing bout frequency for water in the $D2^{ChR2}$ group. These findings indicate that
131 optogenetic activation of NAc D2-expressing neurons disrupts normal licking behavior
132 and induces self-stimulation, suggesting a rewarding effect. This suggests that the
133 observed decrease in sucrose preference likely stems from the combined rewarding and
134 lick-impairing effects of optogenetic stimulation.

## 135 Optogenetic frequency-dependent modulation of licking 136 behavior by NAc D2-expressing neurons

137 To assess the impact of stimulation frequency on licking behavior, we used a freely licking
138 task with a laser scanning frequency test, as previously reported, to examine frequency-
139 dependent activation of D2 NAc neurons.[14] The task operates in a cycle of three
140 epochs: Before (4 licks), Laser (1 s), and Timeout (1 s). A new cycle is triggered by four
141 consecutive licks immediately after the Timeout epoch. In this task, control animals
142 ($D2^{tdTomato}$) exhibited consistent lick rates across all laser frequencies (**Fig 2b**). In contrast,
143 $D2^{ChR2}$ activation reduced the lick rate compared to the No-laser trials, with stronger
144 reductions observed at higher frequencies, especially 8, 15, and 20 Hz (**Figs 2c, 2f, and**
145 **2i**). Cumulative lick distributions during laser epochs revealed significant reductions in the
146 $D2^{ChR2}$ group across all frequencies compared to No-laser trials [# one-way ANOVA; main
147 factor: types of trials; $F_{(4, 625)} = 38.94$, $p < 0.001$, all Tukey- Kramer; $p<0.001$], and the
148 control group ($D2^{tdTomatto)}$ with the most pronounced reductions at 8 Hz [* one-way

149 ANOVA; main factor: groups; $F_{(4, 354)} = 33.98$, $p < 0.001$], 15 Hz [* one-way ANOVA; main
150 factor: groups; $F_{(4, 354)} = 39.93$, $p < 0.001$], and 20 Hz [* one-way ANOVA; main factor:
151 groups; $F_{(4, 354)} = 52.72$, $p < 0.001$] (all $^{\#,@}$ p's < 0.001) (**Fig 2i**). Additionally, the 5 Hz
152 stimulation allowed significantly more licks than higher frequencies (8, 15, and 20 Hz)
153 within the $D2^{ChR2}$ group [one-way ANOVA; 5 Hz vs. other frequencies; $F_{(3, 187)} = 8.79$, $p <$
154 $0.001$]. Inhibition of D2 neurons ($D2^{ArchT}$) at 5 Hz stimulation generated significantly more
155 licks compared to the control group (**Figs 2j**) [*one-way ANOVA; main factor: groups; $F_{(2,}$
156 $_{354)} = 18.69$, $p <0.001$], while there were no significant differences at 8, 15, or 20 Hz
157 compared to the control group (**Fig 2j**). During the Laser epoch, $D2^{ChR2}$ animals displayed
158 significantly fewer licks at 8, 15, and 20 Hz compared to No-laser trials [$^{\#}$one-way ANOVA;
159 main factor: trial types; $F_{(4, 130)} = 14.07$, $p < 0.001$] (**Fig 2k**). Inhibition of D2 neurons
160 ($D2^{ArchT}$) at 20 Hz resulted in significant suppression of licking behavior [$^{\#}$one-way
161 ANOVA; main factor: trial types; $F_{(4, 160)} = 3.44$, $p = 0.009$] (**Fig 2k**). Stimulation effects
162 persisted into the post-laser timeout period, with a tendency for lick frequency to decrease
163 with increasing stimulation frequency (**Fig 2l**). Although this change was less pronounced
164 during the Timeout epoch, the tendency suggests a continued stimulation influence.
165 These results suggest that NAc D2 neuron activity modulates licking behavior in a
166 frequency-dependent manner, acting as a "brake" on consummatory actions and
167 decreasing sucrose intake. Higher stimulation frequencies, particularly at 15 and 20 Hz,
168 produce stronger suppression and have long-lasting effects on reinitiating reward-seeking
169 behavior.

## Self-paced (unlike experimenter-imposed frequency) optogenetic stimulation of D2 neurons attenuates disruption of licking in a brief access taste test

We used the brief access taste test (BATT) to investigate whether the frequency-dependent effects of stimulation on licking behavior persisted under a self-paced lick-driven rhythmic stimulation pattern. In this task, mice could lick for a 10% sucrose (Suc) solution during a 4 s reward period with different optogenetic stimulation trials (No-laser, lick-paired (0 ms delay), 8 and 20 Hz). The $D2^{tdTomato}$ (control) (**Figs 3b, 3e**) group maintained consistent licking behavior across all trials, showing no significant disruption by these modulations. In contrast, based on the optogenetic stimulation pattern, the $D2^{ChR2}$ (activation) group exhibited a significant difference in licking behavior (**Figs 3c, 3f**). Notably, 20 Hz stimulation significantly reduced the licking rate [#one-way ANOVA; main factor; trial types $F_{(4, 416)} = 22.3$, $p < 0.001$], while lick-paired stimulation preserved a licking rate similar to the No-laser condition, highlighting the importance of self-timing in optogenetic stimulation and its effects on licking behavior.

These effects were also reflected in cumulative licks across the session, where the $D2^{ChR2}$ group exhibited significantly fewer licks during stimulation at 20 Hz than the other trial types (**Fig 3f** [#one-way ANOVA; main factor: trial types; $F_{(3, 416)} = 22.63$, $p < 0.001$]). A *post hoc* analysis confirmed that the No-laser and lick-paired conditions significantly differed in the $D2^{ChR2}$ group across trial types [#$p < 0.001$], underscoring the impact of stimulation frequency on licking behavior. The 8 Hz imposed stimulation also reduced cumulative licks compared to the No-laser condition [$p<0.001$], although this reduction

192 was less pronounced than that induced by the 20 Hz stimulation [$^{!}$p<0.001]. While the
193 D2$^{ArchT}$ (inhibition) group significantly increased the cumulative licks in the total licks and
194 in the lick paired, 8, 20 Hz trials (**Figs 3d, 3g**) compared to the control group (all *p's<0.01)
195 (**Figures 3e, 3g**).
196 We plotted the total licks per session to observe the overall impact on licking behavior
197 (**Fig 3h**). This plot further emphasized these differences across groups [*one-way
198 ANOVA; main factor: groups $F_{(2, 65)} = 7.78$, p <0.001]. In the D2$^{ChR2}$ group [*one-way
199 ANOVA; main factor: trial types $F_{(3, 80)} = 7.668$, p <0.001], total licks during the 20 Hz
200 condition were significantly reduced compared to the No-laser($^{\#}$p <0.001) and lick-paired
201 conditions($^{@}$p <0.001) ). Conversely, total licks in the D2$^{tdTomato}$ and D2$^{ArchT}$ groups
202 showed no significant differences across stimulation types, confirming that optogenetic
203 activation of D2-expressing neurons, rather than inhibition, more strongly affected licking
204 behavior.
205 Importantly, the increased licking behavior observed during control trials in the D2$^{ChR2}$
206 group may be explained by the sequential nature of the task. Given that each trial must
207 be completed before passing to the next one, the animals could display an enhanced
208 motivation effect from previous trials where the D2 neurons were activated (panel **h**).
209 Microstructural analysis of licking behavior (**Fig 3i)** revealed, at 8 [*one-way ANOVA;
210 main factor: groups; $F_{(2, 65)} = 12.50$, p <0.001] and 20 Hz [*one-way ANOVA; main factor:
211 groups; $F_{(2, 65)} = 10.03$, p <0.001] frequencies, more fragmented licking in the D2$^{ChR2}$
212 group characterized by shorter bout duration and increased bout number [*one-way
213 ANOVA; group; $F_{(2, 65)} = 16.95$, p < 0.001]. Interestingly, despite the disrupted licking
214 behavior, the D2$^{ChR2}$ group initiated more trials than the other groups (**Fig 3j** [*one-way

215 ANOVA; groups; $F_{(2, 65)} = 14.14$, $p < 0.001$]), indicating that 20 Hz stimulation disrupted
216 consummatory licking but increased the persistence in the task. **Table 3** shows that high-
217 frequency stimulation (20 Hz) significantly reduced lick rate, bout size, and bout duration
218 in the $D2^{ChR2}$ group while increasing the number of licking bouts. This suggests that while
219 high-frequency stimulation disrupts the typical licking behavior, it paradoxically enhances
220 the animal's incentive motivation to persist in seeking the reward, as evidenced by
221 increased trial initiation.

## 222 Lick-paired optogenetic activation with delayed timing of NAc 223 D2 neurons slightly disrupts the licking microstructure but 224 enhances sucrose intake

225 Given the advantage of lick-paired over imposed stimulation, we next assessed the effect
226 of lick-paired but using multiple delays relative to the lick tongue protrusion-retraction
227 cycle. To this aim, we used a variation of the brief access test, where optogenetic
228 activation was applied with different delays relative to the tongue contacting the sipper (0,
229 50, and 100 ms) after each lick, alongside no-laser trials that served as controls. Licking
230 rates remained relatively stable along the reward epoch across all trial types and groups
231 (**Figs 4b-d**), indicating that delayed activation or inhibition had minimal effects on the
232 overall licking behavior. However, despite these stable rates, the $D2^{ChR2}$ group exhibited
233 a significantly higher total number of cumulative licks than the $D2^{tdTomato}$ group across all
234 trial types (**Figs. 4e-f** [*one-way ANOVA; main group: No-laser $F_{(2, 312)} = 11.05$; $p < 0.001$;
235 main group: 0 ms $F_{(2, 312)} = 3.29$, $p = 0.04$; main group: 50 ms $F_{(2, 312)} = 12.49$, $p <0.001$;

236 main group: 100 ms $F_{(2, 312)} = 18.67$, $p < 0.001$; main group: Total cumulative licks $F_{(2, 1257)}$
237 $= 40.15$, $p < 0.001$]. The $D2^{ArchT}$ group also showed significant increases in total
238 cumulative licks (*$p = 0.002$), particularly in the 100 ms delay trials (*$p < 0.001$). These
239 findings indicate a significant increase in the total number of cumulative licks for the
240 $D2^{ChR2}$ group, suggesting greater overall licking activity than the other groups.

241 Although overall, the lick-paired protocol was more 'physiological' than imposed
242 frequency stimulation, we found that the inter-lick interval was significantly longer in the
243 $D2^{ChR2}$ compared to the $D2^{tdTomato}$ group across all conditions (**Fig 4h** [*one-way ANOVA;
244 main factor: group: No-laser $F_{(2, 30)} = 8.35$, $p = 0.01$; 0 ms $F_{(2, 30)} = 13.95$, $p < 0.001$; 50
245 ms $F_{(2, 30)} = 7.57$, $p = 0.002$; 100 ms $F_{(2, 30)} = 13.55$, $p < 0.001$]). This behavioral protocol
246 also revealed the disruptive effect of optogenetic activation on rhythmic oromotor
247 components of licking behavior, even with lick-paired stimulation patterns.

248 Regarding licking bouts, the $D2^{ChR2}$ group showed a significantly higher number of bouts
249 than the $D2^{tdTomato}$ group across all conditions (**Fig 4i** [*all p's < 0.001]). However, the size
250 of each lick bout was smaller in the $D2^{ChR2}$ group compared to the $D2^{tdTomato}$ group under
251 the 0 ms delay condition (**Fig 4j** [*one-way ANOVA; main factor: group $F_{(2,30)} = 6.52$, $p =$
252 $0.01$]).

253 Finally, despite the evident fragmentation of licking behavior, the $D2^{ChR2}$ group initiated
254 significantly more trials compared to both the $D2^{tdTomato}$ and $D2^{ArchT}$ groups (**Fig 4k** [one-
255 way ANOVA; main factor: group; $F_{(2, 30)} = 15.41$, $p < 0.001$]), underscoring a paradoxical
256 increase in task engagement and reward driven by D2 neuron activation. These findings
257 indicate that although self-paced rhythmic optogenetic activation of NAc D2-expressing

258 neurons affects licking microstructure, this effect is less pronounced than when the
259 experimenter imposes the stimulation frequency. Interestingly, this self-paced activation
260 paradoxically increases motivation and task persistence, leading to greater sucrose
261 intake, especially when the activation is paired with physiological licking rhythms.

## 262 Optogenetic activation of NAc D2-expressing neurons at 20 Hz 263 (but not 8 Hz) drives place preference and enhances 264 exploratory behavior

265 Next, we explored how optogenetic activation of NAc D2-expressing neurons modulates
266 spatial contextual rewards beyond licking behavior. We assessed the effects of different
267 stimulation frequencies on place preference using a real-time place preference (RTPP)
268 assay. Optogenetic stimulation was delivered at 8 or 20 Hz in $D2^{ChR2}$ and $D2^{tdTomato}$
269 groups in this paradigm.
270 Heatmaps illustrating the time spent in a spatial location (in yellow; **Figs 5b-e**) revealed
271 a clear preference for the stimulated side in the $D2^{ChR2}$ group when stimulated at 20 Hz
272 (**Fig 5c**). Mice in this group spent significantly more time on the stimulated side during
273 both sessions compared to the $D2^{tdTomato}$ control and the 8 Hz stimulation condition (**Fig**
274 **5f** [*one-way ANOVA; main factor: group; $F_{(3, 22)} = 7.19$, $p < 0.001$]). In contrast, no
275 significant place preference was observed in the $D2^{tdTomato}$, $D2^{ChR2}$ 8 Hz, and $D2^{tdTomato}$
276 20 Hz groups.
277 The number of transitions between the two sides of the chamber did not differ significantly
278 between groups (**Fig 5g**). However, the $D2^{ChR2}$ 20 Hz group exhibited significantly higher
279 velocity on the non-stimulated side compared to other groups(**Fig 5h** [*one-way ANOVA;

280 main factor: group; $F_{(3, 22)} = 8.85$, $p < 0.001$]), and conditions ($^{\#}p = 0.01$) while the total
281 distance traveled was greater on the stimulated side (**Fig 5i** [# t-test (paired); $t(6) = 3.26$,
282 $p = 0.01$]) and against other groups [*one-way ANOVA; main factor: group, $F_{(3, 22)} = 5.36$,
283 $p = 0.006$]. These findings indicate that the increased locomotion was specific to the
284 stimulated chamber, suggesting that the 20 Hz stimulation enhanced both place
285 preference and movement within the stimulated area. These results highlight that 20 Hz
286 stimulation of D2-expressing neurons in the NAc induces a robust rewarding effect, as
287 evidenced by place preference, and modulates locomotor behavior, leading to increased
288 movement within the stimulated environment.

## 289 NAc D2 neuron inhibition induces a negative hedonic state
## 290 (but not aversion) that is unmasked by an HFD choice
## 291 paradigm

292 To investigate the role of NAc D2-expressing neurons in context-dependent behaviors,
293 we first examined their influence on place preference using the RTPP task. Mice freely
294 explored a chamber divided into two distinct halves, with one side paired with optogenetic
295 stimulation. In this task, optogenetic inhibition (continuous stimulation) was delivered to
296 $D2^{ArchT}$ mice, while $D2^{tdTomato}$ controls received 20 Hz stimulation (**Fig 6a**, left; $D2^{tdTomato}$
297 data reused in **Fig 5**). Heatmaps representing animal spatial exploration showed no
298 significant preference for the stimulated side in either group (**Fig 6a**, middle).
299 Quantitatively, the percentage of time spent on the stimulated side was similar across
300 both sessions, with no significant differences between groups (**Fig 6a**, right). These data

301 suggest that silencing NAc D2-expressing neurons generated, *per se,* neither aversion
302 nor preference under this standard RTPP protocol.
303 Next, we assessed the effects of D2 neuron modulation on feeding behavior using an
304 open-loop paradigm and HFD alone in one spatial context. In this protocol, mice were
305 placed in an arena with HFD pellets on both opposite sides, while optogenetic stimulation
306 was applied in 4 s on, 2 s off intervals for $D2^{tdTomato}$ and $D2^{ChR2}$ (20 Hz, 10 ms pulse width)
307 and continuous pulse stimulation for $D2^{ArchT}$ (**Fig 6b**, left). In this context, total HFD intake
308 did not significantly differ between the control ($D2^{tdTomato}$), activation ($D2^{ChR2}$), and
309 inhibition ($D2^{ArchT}$) groups (**Fig 6b**, right), indicating that D2 neuron activation or inhibition
310 *per se* was not sufficient to influence HFD feeding behavior in a neutral environment.
311 Finally, in a variation of the RTPP task, we explored whether NAc D2-expressing neurons
312 modulate place preference and HFD consumption in a conditioned context, where one
313 side of the chamber was paired with optogenetic stimulation, and the mice could choose
314 whether or not to eat HFD pellets on the stimulated side on the box (**Fig 6c**). During
315 conditioning, $D2^{ChR2}$ mice developed a significant preference for the stimulation-paired
316 side, which persisted into the post-conditioning test (**Fig 6d** [all $^{*}$p's <0.006, see
317 Supplementary Statistical Table]). Conversely, $D2^{ArchT}$ mice initially avoided the
318 stimulation-paired side during acquisition [$^{\#}$one-way ANOVA; main factor: sessions; $F_{(4,}$
319 $_{20)}$ = 18.78, $p < 0.02$], but this behavior did not persist in the test phase (**Fig 6d**).
320 Analysis of total HFD intake revealed a tendency for reduced intake in the $D2^{ChR2}$ group
321 during activation, while the $D2^{ArchT}$ group exhibited increased consumption during
322 inhibition (**Fig 6e**). In particular, the $D2^{tdTomato}$ (**Fig 6f**) and $D2^{ChR2}$ (**Fig 6g**) groups had no
323 change in the HFD intake between the stimulated and non-stimulated side, while $D2^{ArchT}$

324 mice significantly increased HFD intake on the non-stimulated side during the final
325 acquisition days, an effect that persisted into the test phase (**Fig 6h** [#one-way ANOVA;
326 main factor: side; $F_{(4,25)} = 4.14$, $p = 0.01$] [t-test; Adq2 $t_{(5)} = -4.40$, $p = 0.007$; Adq3 $t_{(5)} = -$
327 $4.39$, $p = 0.007$]). This suggests that inhibiting NAc D2-expressing neurons induces
328 negative hedonics, increasing palatable food consumption on the non-stimulated side.
329 These findings indicate that NAc D2 neuron inhibition induces a negative hedonic value
330 that an HFD choice test can unmask.

## 331 Inhibition of NAc D2 neuron drives negative reinforcement

332 After establishing that optogenetic activation of NAc D2-expressing neurons induces
333 place preference, we sought to determine whether inhibition of these neurons elicits a
334 negative hedonic value using a negative reinforcement task. In this more sensitive
335 paradigm, mice had to enter an 'off' zone to end optogenetic stimulation delivered in the
336 "on" zone. Stimulation was applied at 20 Hz for the $D2^{ChR2}$ and $D2^{tdTomato}$ groups and
337 continuously applied for the $D2^{ArchT}$ group (**Fig 7a**). Despite the natural tendency of mice
338 to explore, which led to most of their time being spent in the 'on' zone, the $D2^{ArchT}$ group
339 showed a significant shift in behavior, with increased time spent in the 'off' zone compared
340 to the $D2^{tdTomato}$ and $D2^{ChR2}$ groups (**Figs 7b, 7c** [*one-way ANOVA; main factor: groups;
341 $F_{(2, 33)} = 4.27$, $p = 0.03$]), indicating that sustained inhibition of NAc D2-expressing neurons
342 generated a negative valence, prompting mice to avoid the area of stimulation.
343 Representative heatmaps from the third session (**Figs 7d-f**) further illustrate this behavior,
344 emphasizing the negative reinforcement effect of D2 neuron inhibition. These results
345 show that inhibiting NAc D2 neurons elicits a negative hedonic value, with mice actively

346 avoiding areas where stimulation occurs. This active avoidance behavior highlights the
347 role of NAc D2-expressing neurons in driving negative reinforcement.

# 348 Discussion

349 This study demonstrates that dopamine D2 receptor-expressing neurons in the NAc play
350 a dual role in reward processing and ingestive behavior. Optogenetic activation of these
351 neurons at higher frequencies disrupts the licking microstructure, decreasing sucrose
352 preference while paradoxically increasing self-stimulation. This seemingly contradictory
353 effect highlights the complex influence of D2 neuron activity, which can simultaneously
354 enhance incentive motivation while disrupting specific oromotor behaviors. Interestingly,
355 synchronizing stimulation with the animal's natural licking rhythm minimized licking
356 disruptions and promoted sucrose intake. Regarding spatial contextual reward, high-
357 frequency stimulation (20 Hz) was sufficient to drive place preference, confirming the
358 rewarding properties of D2 neuron activation. Conversely, inhibiting these neurons led to
359 a negative hedonic state (but not complete aversion), affecting food choices in specific
360 contexts, particularly when a high-fat diet (HFD) was involved. These findings
361 demonstrate the dual role of NAc D2-expressing neurons in regulating reward and
362 consumption, highlighting the potential of entrained, self-paced stimulation that leverages
363 the naturalistic licking theta rhythm for precise activation of these neurons.
364

365 ## Activation of NAc D2 neurons reduces sucrose preference and
366 increases self-stimulation

367 Optogenetic activation of NAc D2 neurons at 20 Hz disrupted sucrose preference by
368 fragmenting the licking microstructure (**Fig. 1**). This finding is consistent with previous
369 studies emphasizing the role of the NAc in orchestrating consummatory actions and
370 processing palatability-related information.[8] In this regard, electrophysiological and
371 calcium imaging studies in rats and mice have shown that a subset of NAc neurons
372 encodes sucrose concentration and palatability, while other subsets exhibit oromotor
373 responses entrained to the licking rhythm,[1,2,22,23] highlighting that the NAc is
374 embedded in dynamic circuits that regulate hedonic processing and goal-directed
375 behaviors.[7] While D1 neurons have traditionally been associated with feeding,[8,9] our
376 findings indicate that experimentally imposed 20 Hz activation of D2 neurons particularly
377 disrupts ingestive behavior.

378 ## Frequency-dependent disruption of licking behavior by NAc
379 D2-expressing neurons

380 Prado et al. (2016) showed for the first time that optogenetic manipulations of
381 glutamatergic inputs to the NAc are rewarding but also stopped licking behavior in a
382 frequency-dependent manner, reducing lick duration and frequency, resembling our
383 observation of D2 neurons activation shortened lick bout durations and reduced bout
384 sizes (**Fig 2**).[14] Interestingly, these disruptions were frequency-dependent, with higher
385 frequencies (15 and 20 Hz) producing more pronounced effects, supporting the notion

386 that NAc D2-expressing neurons would be, at least, one target of glutamatergic inputs to
387 fine-tune the intensity and timing of consummatory actions.[14]
388 In a detailed frequency test, we found that higher optogenetic stimulation frequencies (15
389 and 20 Hz) produced a significant suppression of licking behavior compared to lower
390 frequencies. These results support that D2-expressing neurons act as modulators of
391 consummatory actions depending on the stimulation pattern, as highlighted by Soares-
392 Cunha et al. (2019). In their study, brief stimulation was rewarding, but prolonged
393 stimulation became aversive and induced more pronounced behavioral impairments.[5]
394 Our study suggests that D2-expressing neurons play a dual role in regulating
395 consummatory behavior. While they enhance the incentive motivation to self-stimulate,
396 they simultaneously impair the execution of licking, as shown by increased self-
397 stimulation despite impaired licking (**Fig 4**). These results also indicate that NAc D2-
398 expressing neurons can modulate ingestive behavior through frequency-dependent
399 mechanisms, aligning with the concept of the NAc as a central hub integrating reward
400 and feeding behaviors, where the activity of D2-expressing neurons can either promote
401 or hinder consumption depending on the specific stimulation pattern.[8,14,15]
402 Our data can also reconcile some differences in literature. For example, O'Connor et al.
403 (2015) found that 20 Hz stimulation of D1 neurons abruptly impaired licking, [9] whereas
404 Sandoval et al. (2023) did not observe licking impairment using a lick-paired self-
405 stimulation protocol of D1 neurons, like the one used in this study. Surprisingly, like us,
406 they also observed a slight licking impairment using lick-paired self-stimulation of D2-
407 expressing neurons, [11] suggesting a unique property of self-rhythmic licking to stimulate
408 the NAc. These results also support the notion that rhythmic licking acts as an internal

clock to synchronize brain activity across different brain regions.[24] While our findings show that D2-expressing neurons impair rhythmic licking, particularly at high frequencies, the precise circuit mechanisms underlying this disruption remain unclear. This is because previous research has shown that lesioning D1, but not D2, neurons impairs licking microstructure,[11] and rabies tracing studies indicate that, unlike D1 neurons, D2 neurons do not directly connect to taste buds or the CPG for licking.[11] One possibility is that D2-expressing neurons disrupt licking behavior indirectly by modulating D1 neuron activity, potentially through lateral inhibition.[25,26] This hypothesis warrants further investigation, which could employ cell-type-specific manipulations and circuit-tracing techniques to delineate the precise pathways and mechanisms involved.

## Self-paced stimulation enhances task engagement and reduces licking disruption

One of the key insights from the present study is the better preservation of licking behavior and enhanced task engagement when stimulation was synchronized with natural licking rhythms (**Figs 3,4**), highlighting the flexible nature of reward-seeking behaviors governed by the NAc. This finding aligns with previous research showing that mice will self-stimulate glutamatergic inputs to the NAc and that these glutamatergic inputs are suppressed during feeding.[14,27] This suggests that NAc circuits dynamically adapt to regulate both consumption and the pursuit of rewards.[13,14,27] Similarly, Cole et al. (2018) reported that allowing animals to self-stimulate D2 neurons by licking a spout, mirroring their natural behavior, led to increased engagement and fewer disruptions compared to externally imposed stimulation triggered by entering a specific spatial location.[17] These

431 findings resonate with the concept that D2 neuron activity can modulate reward by
432 integrating motor information into reward-seeking,[28] promoting goal-directed behaviors
433 while maintaining hedonic value.[1] This supports the view that stimulation parameters,
434 particularly self-paced stimulation, are crucial in determining the behavioral outcomes of
435 D2 neuron activation.[4]

## 436 High-frequency activation of NAc D2 neurons drives place 437 preference and enhances exploratory behavior

438 In addition to modulating licking behaviors, our study revealed that high-frequency
439 activation of NAc D2 neurons (20 Hz) also induced robust place preference and
440 significantly enhanced exploratory behavior, indicating a strong, rewarding effect (**Fig 5**).
441 While place preference has traditionally been associated with D1 neurons,[20] our results
442 extend these findings to D2-expressing neurons, indicating that these neurons play a
443 more significant role in reward-seeking behaviors than previously assumed. Our data also
444 supports the observation of Cole et al. (2018) using a lick-spout self-stimulation task. They
445 found that mice self-stimulated both D1 and D2 neurons in NAc, although D1 excitation
446 was more rewarding than D2 neurons. They also suggest that the excitation of D2
447 neurons in NAc supports self-stimulation under some tasks (using a lick-spout) but fails
448 under others.[17] Oginsky et al. (2016) showed that NAc D2-expressing neurons are
449 involved in both place preference and exploratory behaviors, particularly in contexts
450 involving palatable food, supporting our findings of enhanced exploratory drive during 20
451 Hz stimulation.[29]

452 The enhanced exploratory behavior observed in the stimulated context suggests that D2-
453 expressing neurons may contribute to a broader behavioral repertoire beyond reward.[15]
454 This indicates a potential role for D2-expressing neurons in modulating spatial exploration
455 and context-dependent behaviors, contributing to the animal's overall motivational state
456 during task engagement.[26]

## 457 Inhibition of NAc D2 neurons decreases palatable food intake
## 458 and drives context-dependent negative reinforcement

459 Interestingly, while optogenetic activation produced place preference, inhibition of D2
460 neurons also revealed a role in processing hedonic value (**Fig 6**). Specifically, inhibition
461 led to a preference for HFD consumption on the non-stimulated side of a conditioned
462 chamber, suggesting that D2 neuron suppression induces a negative hedonic value that
463 palatable food can partially mask. Yang et al. (2020) found that inhibition of D2 neurons
464 unmasked negative hedonic value in specific contexts, mirroring our findings that D2
465 inhibition during HFD reveals a negative hedonic signal that palatable food can
466 override.[3] Cole et al. (2018) reported that optogenetic activation of D2 neurons leads to
467 a motivational state where both reward-seeking and sometimes negative avoidance
468 responses coexist, suggesting that D2-expressing neurons exhibit a context-dependent
469 ambivalence response.[17] These findings are consistent with earlier studies
470 demonstrating that D2 neurons contribute to positive and negative valence processing
471 depending on the context and task structure.[4,5] This result emphasizes the context-
472 dependent nature of D2 neuron function, where inhibition generates a negative hedonic
473 state, further supporting the idea that D2-expressing neurons can induce a negative

474 reinforcement (**Fig 7**). Our findings extend the current understanding of NAc D2-
475 expressing neurons, showing their critical role in balancing reward and consummatory
476 behavior across diverse contexts of motivation and motor control. This duality highlights
477 their complex contribution to reward circuits, aligning with the broader literature on their
478 dual functions.[15,17,30]

## 479 Limitation of the study

480 Although our findings provide new insights into the role of NAc D2-expressing neurons in
481 reward and consummatory behaviors, it is important to acknowledge some limitations.
482 Firstly, while this study focused on D2-expressing neurons, future research should
483 investigate the role of D1-expressing neurons with similar paradigms to understand better
484 the network dynamics governing these behaviors. Future work should explore how D1
485 and D2 neurons interact to regulate both homeostatic and hedonic feeding, building upon
486 work by Walle et al. (2024), using simultaneous manipulations of D1 and D2 expressing
487 neurons that highlighted distinct but complementary roles for these neurons in energy
488 balance and reward-seeking.[15] Secondly, while we observed significant frequency-
489 dependent effects, the precise circuits and downstream targets mediating these effects
490 require further investigation. It is important to recognize that our use of optogenetics to
491 modulate NAc D2-expressing neurons (or any other cell type) while providing valuable
492 insights may not fully capture the 'physiological' role of endogenous neural activity and
493 naturalistic reward behaviors.[31] This study found that behavioral responses are highly
494 dependent on optogenetic stimulation parameters. Therefore, chemogenetic
495 manipulations are also expected to produce varying behavioral effects as different

496 stimulation approaches engage neuronal circuits in distinct ways.[15] The observation
497 that different stimulation parameters of D2-expressing neurons result in different
498 behavioral outputs aligns with the concept of optoception. We recently reported the
499 optoception phenomenon, suggesting that mice could perceive each stimulation pattern
500 slightly differently, providing a basis for mice's ability to identify and discriminate between
501 various optogenetic stimulation frequencies.[31] Finally, because cholinergic
502 interneurons in the NAc also express D2 receptors, future studies employing
503 intersectional genetic techniques are needed to differentiate the contribution of these
504 interneurons from those of D2 MSNs. This will provide a more precise understanding of
505 the specific roles of these distinct cell populations in reward and consumption.

## 506 Conclusion

507 In summary, our findings demonstrate the dual role of NAc D2-expressing neurons in
508 modulating reward and consummatory behavior, where activation can simultaneously
509 enhance incentive motivation to self-stimulate and disrupt licking behavior. These results
510 highlight the importance of stimulation parameters, frequency, timing, and agency of
511 actions in shaping the behavioral outcomes of D2 neuron activity. Inhibition of these
512 neurons induces a negative hedonic state that is (un)masked in specific contexts, such
513 as during HFD consumption. Thus, NAc D2-expressing neurons act as critical
514 modulators, balancing reward and licking disruption through rhythmic optogenetic
515 stimulation, providing new insights into their role in reward circuits. These findings deepen
516 our understanding of the NAc role in reward circuits and may provide potential avenues

517 for exploring therapeutic interventions for disorders related to reward processing, such as
518 addiction[32] or obesity.[33]

# 519 Materials and methods

## 520 Ethical Considerations

521 All experimental procedures were approved by the Cinvestav Animal Care and Use
522 Committee (CINVESTAV) under permit number [0080-14] and conducted the Guide for
523 the Care and Use of Laboratory Animals of the National Institutes of Health.

## 524 Animals

525 Drd2-Cre (B6.FVB(Cg)-Tg(Drd2-cre)ER44Gsat/Mmcd) mice of both sexes (7-10 weeks
526 old, 20-25 g at the beginning of the experiment; MMRC, stock number: 032108) were
527 single-housed in a temperature-controlled room (22 ± 1°C) with a 12-h light/dark cycle.
528 Mice had *ad libitum* access to a standard chow diet (PicoLab Rodent Diet 20, MO, USA)
529 and water unless otherwise specified for a particular behavioral paradigm. After surgery,
530 mice were single-housed and maintained in a temperature-controlled environment with a
531 12-h light/dark cycle. Post-surgical care included the administration of analgesic
532 (lidocaine hydrochloride) and antibiotic (enrofloxacin). Mice were monitored daily for signs
533 of pain, distress, or discomfort to ensure animal welfare. Efforts were made to reduce the
534 number of animals used by implementing habituation protocols and non-invasive
535 techniques, such as video tracking systems for behavioral monitoring.

536 ## Viral constructs

537 For cell-type-specific manipulation of D2-expressing neurons in the NAc, mice were
538 injected with Cre-dependent adeno-associated viral (AAV) vectors carrying genes
539 encoding Channelrhodopsin (ChR2) for optogenetic activation and Archaerhodopsin
540 (ArchT) for neuronal inhibition, as well as control vectors like tdTomato. AAV vectors were
541 obtained from Addgene (www.addgene.org): ChR2-eYFP (AAV5-EfIa-
542 DIOhChR2(E123T/T159C)-EYFP, #35509) at a titer of 1 x 1013 vector genome/ml
543 (vg/ml); ArchT-tdTomato (AAV5/FLEX-ArchT-tdTomato, #28305) at 7.0 x 1012 vg/ml;
544 eYFP-vector (AAV5-EfIa-DIO EYFP, #27056) at 1.0 x 1013 vg/ml; and AAV5 pCAG-
545 FLEX-tdTomato-WPRE (#51503) at 3.8 x 1013 vg/ml. Viruses were aliquoted and stored
546 at -80°C until use.

547 ## Stereotaxic surgery, viral infection, and fiber implantation

548 Mice were anesthetized with 2% isoflurane (flow rate 0.5 L/min) and prior to surgery, mice
549 received a subcutaneous analgesic (lidocaine hydrochloride; 5 mg/kg) on the scalp and
550 topical ophthalmic ointment for eye protection. The scalp was shaved and disinfected with
551 aseptic cotton swabs. Mice were then placed in a stereotaxic frame, and a midline sagittal
552 scalp incision (~1 cm) was made to expose the skull, which was leveled in the bregma-
553 lambda plane. Two stainless steel holding screws were implanted bilaterally onto the
554 skull. A two-step procedure involving viral infection and fiber implantation was performed
555 to achieve targeted gene expression and subsequent optical stimulation within the
556 NAcSh. A microinjection needle (30-G) connected to a 10-µl Hamilton syringe was used
557 for bilateral injections of AAV targeting the NAcSh at the following coordinates from

558 bregma (mm): AP +1.4, ML ± 0.65, DV -4.4. The injection volume was 250 nL at a rate of
559 30 nL/min, and the injector was left in position for an additional 5-min period to allow for
560 virus diffusion. Although the intended region was the NAcSh, some virus spread occurred
561 in the core region. Therefore, we refer to this manipulation as targeting the anterior medial
562 NAc. After AAV injection, a zirconia ferrule with a multimode optical fiber (200 μm,
563 Thorlabs) was implanted into the NAcSh at the following coordinates from bregma (mm):
564 AP +1.4, ML ± 1.35, DV -3.9, with a 10° angle (Stoelting stereotaxic instrument Model
565 Number 51730). The antibiotic enrofloxacin (5 mg/kg, i.p.) was administered once daily
566 for 3 days post-surgery. Mice were allowed to recover for 3 weeks to obtain stable
567 expression of ChR2, ArchT, EYFP, or tdTomato. In the $D2^{tdTomato}$ control group,
568 stimulation parameters were pseudo-randomly selected to match the protocols used for
569 the $D2^{ChR2}$ and $D2^{ArchT}$ groups, ensuring that control mice were exposed to similar
570 conditions without active modulation of D2 neuron activity.

## 571 Optogenetic parameters

572 Mice expressing ChR2, ArchT opsins, or tdTomato were stimulated with a diode-pumped
573 solid-state laser system with a light output intensity of 10 mW at the tip of the optical fiber
574 patch cord, as measured with an optical power meter (PM20A, Thorlabs). For the $D2^{ChR2}$
575 group, the laser output wavelength was 473 nm (OEM Laser) with a pulse duration of 10
576 ms delivered at 20 Hz (unless otherwise indicated for each behavioral paradigm). For the
577 $D2^{ArchT}$ group, the laser output wavelength was 532 nm (Laserglow Technologies), and
578 continuous light pulses were delivered (unless otherwise indicated). The tdTomato-

579 expressing mice received random stimulation parameters from either the ChR2 or ArchT
580 protocols.

## 581 Gustatory stimuli

582 Liquid solutions: Reagent-grade sucrose (Sigma-Aldrich) was diluted in distilled water to
583 yield 3%, 10%, and 18% w/v sucrose solutions. Solutions were prepared fresh every other
584 day, stored overnight at 4°C, and brought to room temperature (~23°C) before use. Solid
585 diets: For solid food consumption experiments, two types of food were provided: a
586 standard chow diet (PicoLab Rodent Diet 20) and a HFD with 45% kcal fat (Research
587 Diets).

## 588 Behavioral tasks

589 All behavioral tasks were automated using an operant conditioning controller (Med
590 Associates Inc., VT, USA). Lick events were recorded with a contact lickometer (Med
591 Associates Inc., VT, USA). Mice were water-restricted for 23 h before the session and
592 given access to water for 1 h afterward for tasks involving licking behavior. For food
593 consumption tasks, mice were allowed to eat only a standard chow diet corresponding to
594 10% of their body weight (2-3 g) between sessions. For tasks requiring training, mice
595 were habituated to the patch cord during training sessions by connecting them to the
596 patch cord without laser stimulation.

## Two-bottle free-licking closed-loop assay

Mice were habituated to the behavioral box (43 x 23 cm) for two days before training. The training session aimed to familiarize mice with the two-bottle setup and establish similar licking behavior from both bottles. The box was equipped with two water bottles, with gravity-fed sippers, and connected lickometers on each side of the frontal wall. The entire test lasted six days, with one 20-min session per day. The positions of the water and sucrose bottles were randomized and counterbalanced between sessions across subjects. The procedures implemented each day were: a) Baseline (Days 1-2): Mice freely licked from both bottles without optogenetic stimulation to establish baseline sucrose preference. b) Laser Sucrose (Days 3-4): Licking the sucrose bottle triggered optogenetic stimulation. c) Laser Water (Days 5-6): Licking the water bottle triggered optogenetic stimulation. Optogenetic stimulation was delivered in a 4-s-on and 2-s-off cycle as long as the mice continued licking. The sucrose preference score was calculated as the ratio of licks received by the sucrose bottle divided by the total licks from both bottles for each day.

## Sucrose freely licking with optogenetic frequency scan

On the day before the experiment, mice were habituated to a behavioral box (22 x 18 cm, Med Associates). The task comprised five consecutive daily 20 min sessions. Each trial included three epochs: Before (laser off): The mouse had to complete three licks to initiate a new trial and enter the laser epoch. Laser epoch: On the fourth lick, the laser was activated for 1 s at different stimulation frequencies, presented in pseudo-random order: no stimulation (control) or 10 ms pulse width at 5, 8, 15, or 20 Hz. This ensured that the

619 animal could not predict the stimulation frequency. Time out (laser off): During this 1-s
620 epoch following laser stimulation, the laser could not be reactivated. The next trial began
621 after the mouse completed three additional licks following the time-out epoch. Each lick
622 was rewarded with a drop of sucrose solution (10% w/v, ~2 μL) from a licking spout
623 throughout the task, and the frequency-dependent effects of optogenetic stimulation on
624 licking behavior were analyzed.

## 625 Brief access taste test with different optogenetic stimulation 626 frequencies

627 Building upon earlier investigations, this variation of the brief access task examined the
628 effect of different stimulation frequencies (during the reward window) on motivated licking
629 behavior. The same behavioral box (22 x 18 cm, Med Associates) was used. The task
630 consisted of five consecutive daily 20 min sessions, each involving trials initiated by a lick
631 on the sipper. Following the lick, a 4-s reward epoch began, signaled by an auditory cue.
632 Every lick triggered the delivery of 10% w/v sucrose solution during this reward window.
633 Simultaneously with sucrose reward delivery, a laser was activated at different
634 frequencies in a pseudorandom order: no stimulation (control), 10 ms pulse width lick-
635 paired, 8 Hz, or 20 Hz. After the reward window ended, subsequent licks were not
636 rewarded. To initiate a new trial, mice had to stop licking for a randomly chosen inter-trial
637 interval (ITI) between 1 and 3 s.

## 638 Brief access test with varying delays of optogenetic stimulation

639 This variation of the brief access task assessed the effect of the timing of lick-paired
640 optogenetic stimulation on behavior. The overall structure was like the previous brief

641 access task, with the key difference being the timing of the laser stimulation.
642 Simultaneously with sucrose reward delivery, a laser was activated at different delay
643 times in a pseudo-random order: no stimulation (control) or a 10 ms pulse delivered at
644 specific delays after the lick (lick-evoked), with no delay (0 ms), a 50 ms delay, or a 100
645 ms delay.

## 646 Real-time place preference (RTPP)

647 Mice were placed in a rectangular acrylic arena (42 x 20 x 20 cm) divided into two halves
648 differentiated by context (visual cues: one side with black/white stripes, the other with
649 black/white circles; Fig 5a). Mice freely explored both sides of the chamber. The
650 experiment included two 10-min sessions separated by approximately 5 h on the same
651 day. During each session, one side of the chamber was paired with optogenetic
652 stimulation. Each Time mice crossed to the paired chamber; they received 4 s of
653 optogenetic stimulation followed by 2 s without stimulation. This cycle (4 s on, 2 s off)
654 could repeat as long as the mouse remained on the paired side. The stimulation
655 parameters were: $D2^{tdTomato}$ and $D2^{ChR2}$, 10 ms pulse at 20 Hz and 8 Hz stimulation (each
656 frequency used on a separate day); $D2^{ArchT}$: Continuous pulse stimulation. The preference
657 index for the conditioned and unconditioned sides was calculated by dividing the total
658 time spent on each side by the total exploration time of the session. The chamber side
659 paired with optogenetic stimulation was counterbalanced across subjects.

## 660 Open-loop palatable (HFD) consumption test

661 This task investigated how mice respond to HFD consumption without context
662 conditioning elements. Mice were placed in a white arena with the same dimensions as

663 in the RTPP task, with an HFD pellet on each side (pellet weight measured before and
664 after). Mice freely explored for a single 20-min session, receiving stimulation: 4 s on, 2 s
665 off ($D2^{tdTomato}$ and $D2^{ChR2}$, 20 Hz, 10 ms pulse; $D2^{ArchT}$, Continuous pulse). Using the same
666 stimulation parameters as RTPP isolated the effects of optogenetic stimulation on HFD
667 consumption from context conditioning. Food consumption was calculated by dividing the
668 pellet weight difference by session time.
669

670 **RTPP with palatable (HFD) consumption**

671 Similar to the RTPP task, mice were placed in a rectangular acrylic arena (same
672 dimensions) with an HFD pellet placed on each side. The pellet weight was determined
673 before and after each session. Mice freely explored both sides. The experiment consisted
674 of five consecutive daily sessions: a) Pre-Test (Day 1, 10 min): Mice explored with HFD
675 pellets but no optogenetic stimulation; b) Acquisition (Days 2-4, 20 min): Based on the
676 pre-test exploration time, mice were assigned a conditioned side (more Time for $D2^{tdTomato}$
677 and $D2^{ArchT}$ or less preferred for $D2^{ChR2}$). When crossing to and remaining on the
678 conditioned side, they received optogenetic stimulation: $D2^{tdTomato}$ and $D2^{ChR2}$: 4 s on (20
679 Hz, 10 ms pulse), 2 s off; $D2^{ArchT}$: Continuous pulse; c) Test (Day 5, 10 min): Mice
680 explored with HFD pellets without stimulation. The preference index was calculated by
681 dividing the time spent on each side by the total exploration time. Food consumption was
682 calculated by dividing the pellet weight difference by session time.

683 ## Negative reinforcement test

684 Mice were tested for negative reinforcement in a white open circular arena (50 cm
685 diameter) where they could freely explore during daily 30-min sessions for 3 consecutive
686 days. Behavior was monitored from above using a video camera, and the mouse's
687 position was analyzed in real-time using custom MATLAB code. The code calculated the
688 centroid of the mouse and sent this information to the operant conditioning controller (Med
689 Associates Inc., VT, USA). The arena was virtually divided into two zones: an inner circle
690 (10 cm diameter) designated as the 'off zone' and an outer annulus surrounding the off
691 zone defined as the 'on zone.' The on zone was paired with optogenetic stimulation, and
692 when a mouse entered the on zone, it received 4 s of optogenetic stimulation followed by
693 2 s without stimulation. This cycle could be repeated if the mouse remained in the on
694 zone. The stimulation parameters were $D2^{tdTomato}$ and $D2^{ChR2}$, 10 ms pulse at 20 Hz;
695 $D2^{ArchT}$, optogenetic stimulation was delivered as continuous pulses. To stop optogenetic
696 stimulation, the mouse had to enter the off zone and remain there. The analysis software
697 determined zone entry and exit based on the position of the calculated centroid provided
698 by the MATLAB code. The percentage of time spent in the stimulated and unstimulated
699 zones was calculated by dividing the total time spent in each zone by the total exploration
700 time of the session.
701

702 # Histology

703 After the experimental sessions, mice received a pentobarbital sodium overdose and
704 were transcardially perfused with a phosphate-buffered saline solution followed by 4%

705 paraformaldehyde (PFA). Brains were removed, stored for 1 day in 4% PFA, and then
706 transferred to 30% sucrose in phosphate-buffered saline solution. Brains were sectioned
707 into 40-μm coronal slices and mounted in a medium (Dako) for confocal microscopy to
708 confirm viral expression and fiber placement. Mice with incorrect expression or fiber
709 placement were excluded from the analysis. Images were taken with a confocal
710 microscope, and contrast was improved with Adobe Photoshop CS5.1 software.

## 711 Quantification and statistical analysis

712 Data were analyzed in MATLAB R2024a (The MathWorks Inc.) and GraphPad Prism 9.
713 Data were expressed as means ± SEM. Statistical analyses were performed by first
714 assessing normality distribution, homoscedasticity, and sample size to decide the use of
715 parametric or non-parametric tests. As most of the data met the assumptions for
716 parametric tests, one-way ANOVA was used, followed by a Tukey-Kramer post hoc test
717 with an $\alpha$ level of 0.05. Sample sizes were decided based on previous studies using
718 similar behavioral paradigms and are reported in figure legends. Potential confounders,
719 such as variations in individual animal behavior and environmental factors, were
720 controlled by randomizing the order of experimental conditions and balancing the groups
721 by sex and age.

# 722 Data Availability Statement

723 All relevant data are within the paper and its Supporting Information files. Additional data
724 is available on request from the corresponding author.

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# Competing Interests

The authors have declared that no competing interests exist.

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# Figures

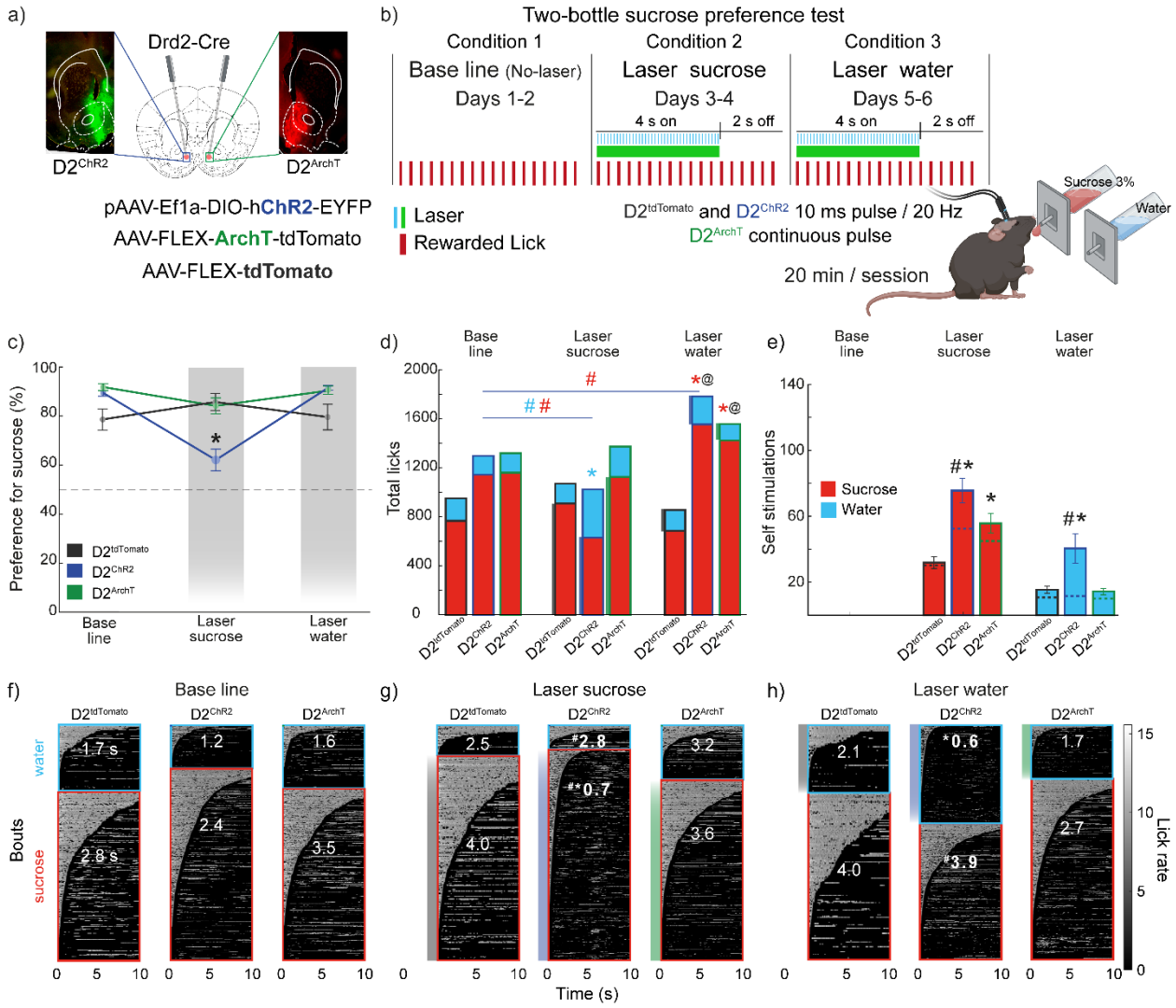
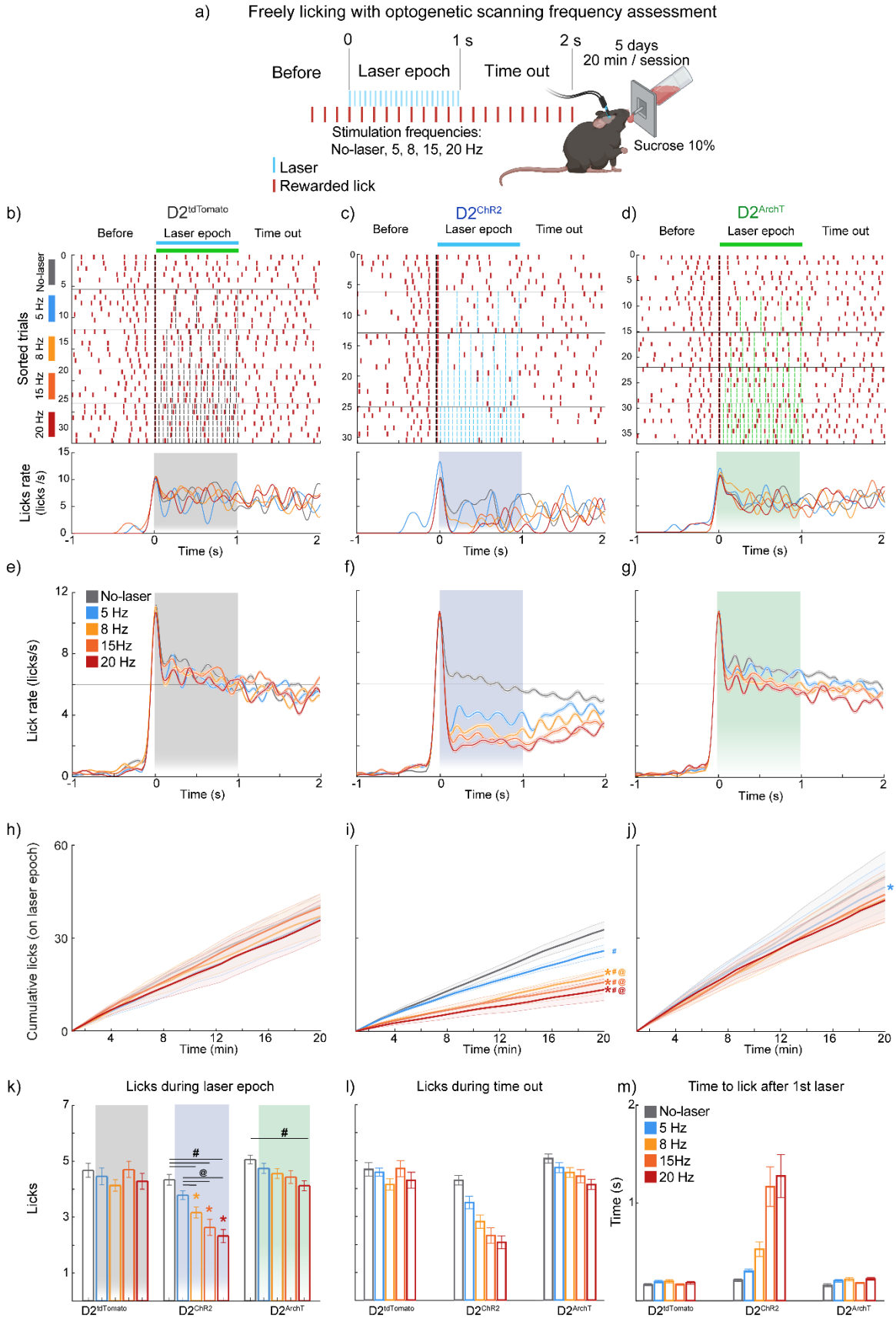



**Figure 1. Optogenetic activation of NAc D2 neurons at 20 Hz reduces sucrose preference, disrupts licking microstructure, and increases self-stimulation trials.**

(**a**) Coronal brain sections depicting Cre recombinase expression in D2-expressing neurons. Below, the viral constructs used: pAAV-Ef1a-DIO-hChR2-EYFP (activation), AAV-FLEX-ArchT-tdTomato (inhibition), and AAV-FLEX-tdTomato (control). (**b**) Two-bottle test protocol: baseline (No-laser, Days 1-2), laser triggered by licking the sucrose

847 bottle (Days 3-4), and laser triggered by the water bottle (Days 5-6). (**c**) Sucrose
848 preference (%) across conditions in each group. (**d**) Total licks during baseline, laser
849 sucrose, and laser water conditions. (**e**) Total trials (number of trials with self-activated
850 laser). Dashed lines represent the baseline, calculated from the licks given per each
851 solution in the off condition and the trial time (4 seconds on, 2 seconds off), which reflects
852 the expected times the mice would start a new trial if laser stimulation were present. (**f-h**)
853 Heatmaps of licking bouts. Water-licking bouts are shown in blue, and sucrose-licking
854 bouts in red. (**f**) Baseline condition. (**g**) 4 s laser triggered with sucrose bottle. (**h**) Laser
855 triggered with a water bottle. Licking rate (licks per second) is shown on the right.
856 * significant differences with the control group ($D2^{tdTomato}$) within the same condition; #
857 significant differences compared to baseline within the same group. @ significant
858 difference for total licks (water + sucrose) against control group within the same condition.
859 All significant differences had $p < 0.05$. Data are mean ± SEM. The statistical analyses
860 were conducted with one-way ANOVA followed by Tukey-Kramer post hoc tests.
861 $D2^{tdTomato}$ n=5, $D2^{ChR2}$ n=11, $D2^{ArchT}$ n=9. Licking microstructure details are provided in
862 **Table 1**.
863


a) Freely licking with optogenetic scanning frequency assessment
0
1 s
2 s
5 days
20 min / session
Before
Laser epoch
Time out
Stimulation frequencies:
No-laser, 5, 8, 15, 20 Hz
Sucrose 10%
Laser
Rewarded lick
b) D2tdTomato
c) D2ChR2
d) D2ArchT
Before
Laser epoch
Time out
Sorted trials
No-laser
5 Hz
8 Hz
15 Hz
20 Hz
Licks rate (licks /s)
Time (s)
e)
f)
g)
Lick rate (licks/s)
No-laser
5 Hz
8 Hz
15Hz
20 Hz
h)
i)
j)
Cumulative licks (on laser epoch)
Time (min)
k) Licks during laser epoch
l) Licks during time out
m) Time to lick after 1st laser
Licks
Time (s)

865 **Figure 2. Optogenetic activation of NAc D2-expressing neurons disrupts licking**
866 **behavior in a frequency-dependent manner.**

867 (**a**) Schematic representation of the freely licking test with 10% sucrose and an
868 optogenetic scanning frequency. The freely licking task is composed of three epochs:
869 Before (four licks), Laser (1 s), and Time out (1 s). Every four licks given after the time-
870 out epoch starts a new Laser epoch. (**b-d**) Representative raster plots showing individual
871 licks (red ticks) and laser pulses (blue for activation, green for inhibition) during a single
872 session in $D2^{tdTomato}$ (control), $D2^{ChR2}$ (activation), and $D2^{ArchT}$ (inhibition) groups. The
873 figures below correspond to the Peri-Stimulus Time Histogram (PSTH) of the single
874 session. (**e-g**) Population PSTHs display mean lick rates for all mice aligned to laser
875 stimulation onset (Time = 0 s). Note that the licking structure is disrupted in a frequency-
876 dependent manner. (**h-j**) Cumulative lick distributions during laser epochs across the
877 session confirmed the disruption of licking for the $D2^{ChR2}$ group. (**k**) Total licks during the
878 1 s laser epoch reveal frequency-dependent disruption in the $D2^{ChR2}$ group. (**l**) Licks
879 during the 1 s timeout phase (post-laser) show recovery. (**m**) The time to start licking after
880 the first laser pulse (Time = 0 s) shows delayed re-initiation in the $D2^{ChR2}$ group, increasing
881 with frequency. * significant differences from the control group ($D2^{tdTomato}$) within the same
882 condition; # significant differences compared to No-laser, @ significant differences
883 compared to 5 Hz frequency within the same group. All significant differences had $p <$
884 $0.05$. Data are mean ± SEM. The statistical analyses were conducted with one-way
885 ANOVA followed by Tukey-Kramer post hoc tests. $D2^{tdTomato}$ n=3, $D2^{ChR2}$ n=8, $D2^{ArchT}$
886 n=7. Licking microstructure details are provided in **Table 2**.

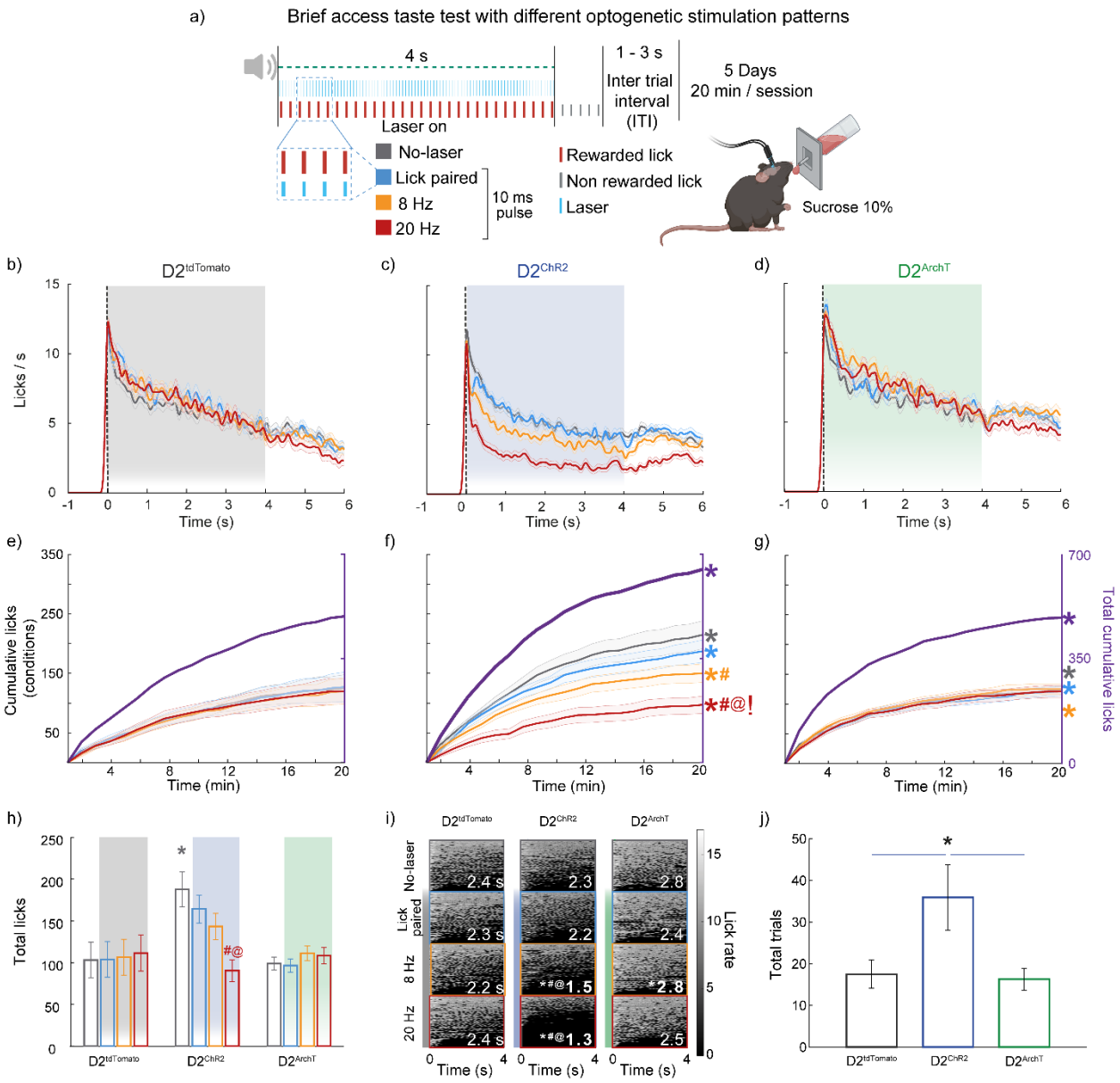
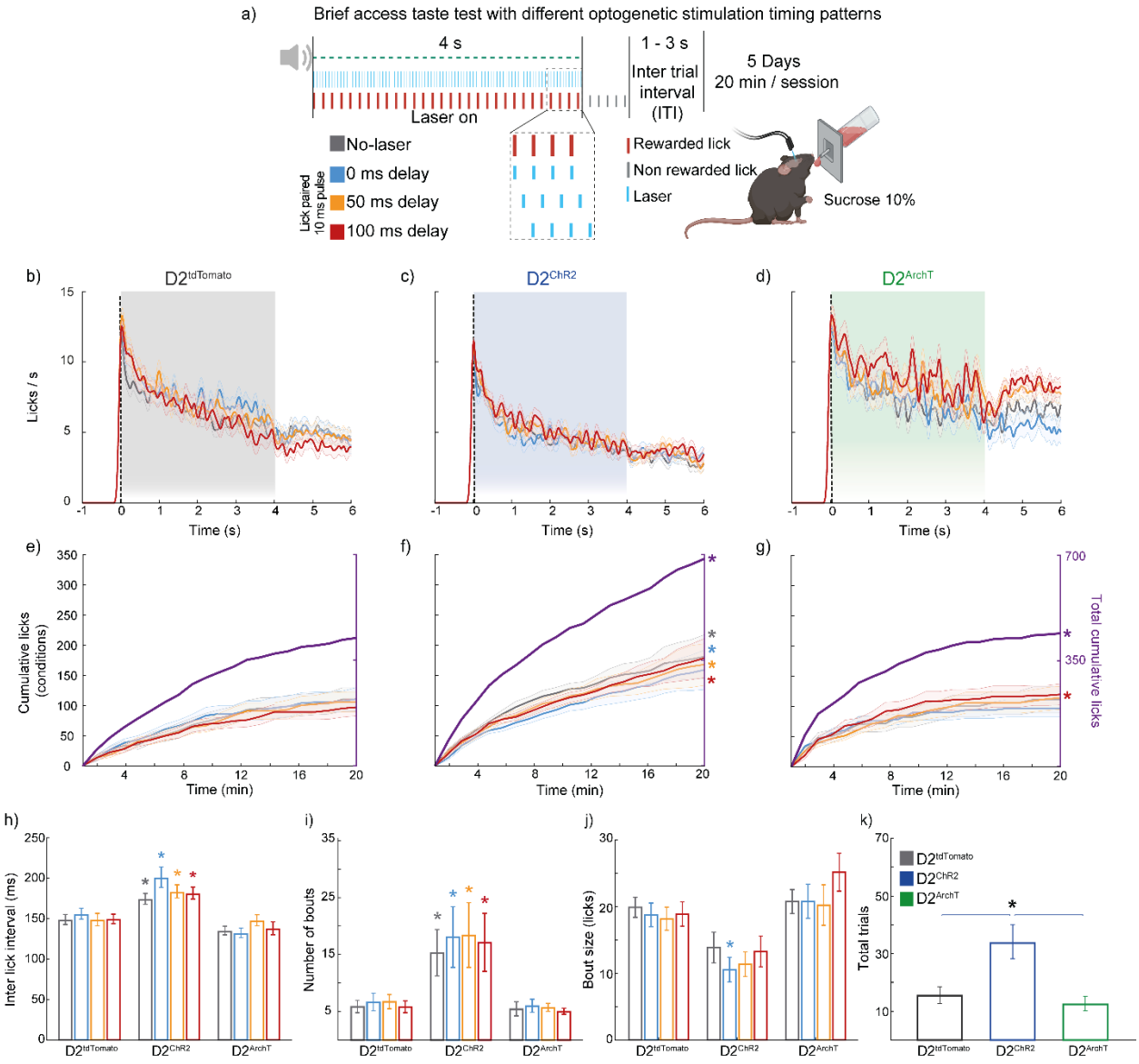



**Figure 3. Frequency-dependent disruption of licking behavior in a sucrose brief access test demonstrates the advantage of self-paced (lick-paired) activation over imposed optogenetic stimulation and exhibits enhanced task engagement through D2 neuron activation.**

(**a**) Schematic representation of the sucrose brief access (4 s) taste test with optogenetic stimulation, including No-laser (gray) and 10 ms pulses at 0 ms delay (blue), 8 Hz (yellow),

894 and 20 Hz (red) frequencies for 4 s. Each trial included the delivery of sucrose in each
895 lick for 4 s (red ticks); after that, additional licks were empty (gray ticks). To start a new
896 trial, mice must stop licking for a 1-3 s inter-trial interval (ITI). The experiment comprises
897 5 sessions, each lasting 20 min. (**b-d**) PSTH displaying mean sucrose lick rates during
898 the 6 s of trial for all $D2^{tdTomato}$ (**b**), $D2^{ChR2}$ (**c**), and $D2^{ArchT}$ mice **(d**). The shaded area
899 represents the 4 s laser stimulation period. (**e-g**) Cumulative licks over the 20-minute
900 session for all stimulation patterns within the same group are shown on the left y-axis,
901 with total cumulative licks (in purple) on the right y-axis. (**h**) Total licks per session for
902 each group under different stimulation conditions. (**i**) Heatmaps plotting licking bouts
903 across stimulation patterns for all groups, with the x-axis representing time in s (0-4 s)
904 from lick bout onset and the y-axis showing individual bouts (the number in white indicates
905 lick bout duration in s). Dark gray edges (bar line at left) represent No-laser bouts, blue
906 edges lick-paired laser stimulation, yellow edges 8 Hz imposed stimulation, and red edges
907 20 Hz stimulation. Light gray intensity indicates lick rate. (**j**) Total trials given by each
908 group under different stimulation conditions. The activation of D2 neurons in the NAc led
909 to significantly more trials started in the $D2^{ChR2}$ group than in the $D2^{tdTomato}$ group. Colored
910 symbols: * significant difference compared to $D2^{tdTomato}$ at the same frequency, #
911 significant difference compared to the No-laser condition within the same group, and @
912 significant difference between the lick-paired condition and the 8 Hz or 20 Hz conditions
913 within the same group. The symbol ! indicates a significant difference between the 8 Hz
914 and 20 Hz conditions within the same group. All significant differences had $p < 0.05$. Data
915 are mean ± SEM. The statistical analyses were conducted with one-way ANOVA followed

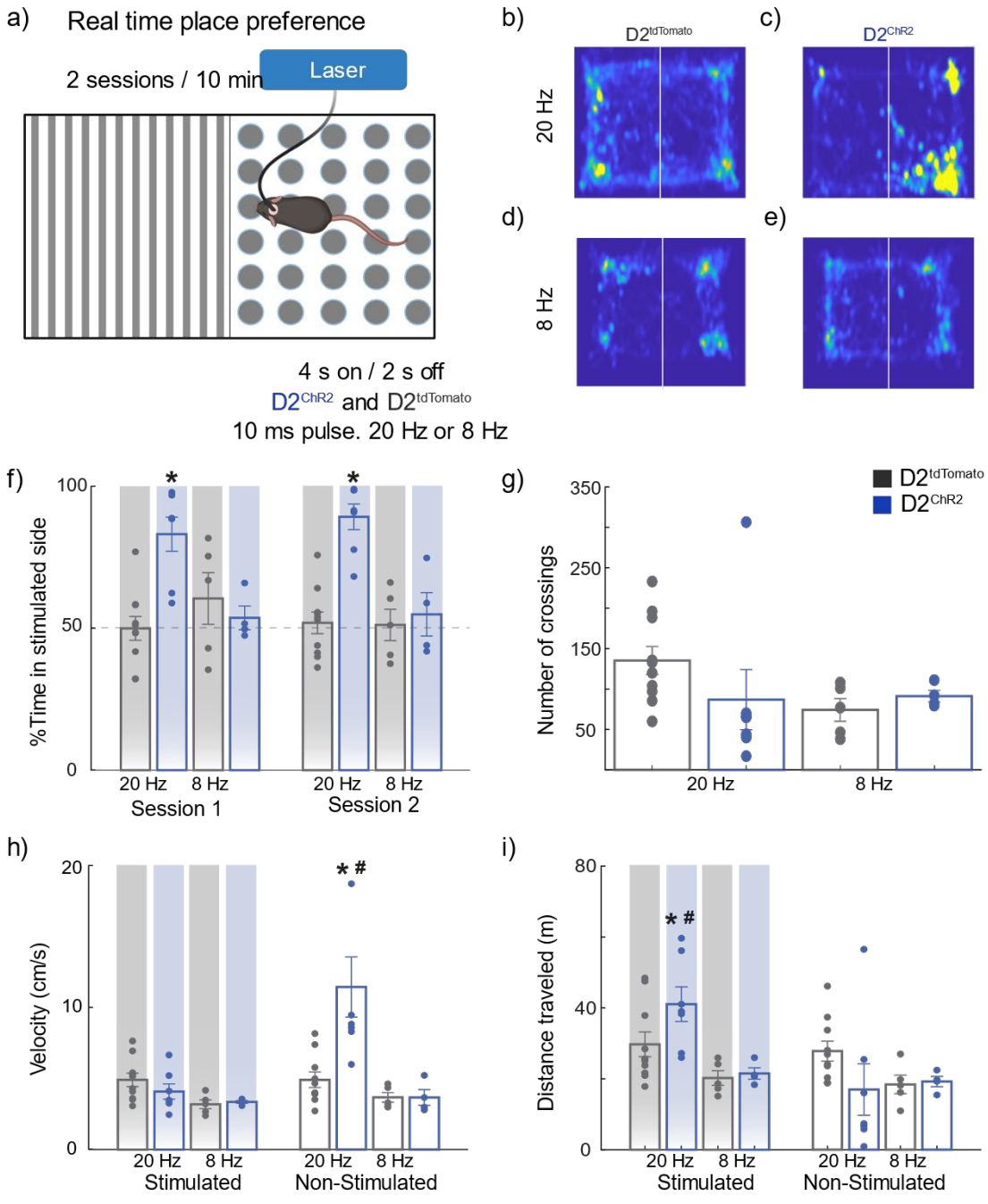
by Tukey-Kramer post hoc tests. $D2^{tdTomato}$ n=6, $D2^{ChR2}$ n=5, $D2^{ArchT}$ n=5. Further analysis of the licking microstructure is presented in **Table 3**.



920 **Figure 4. Lick-paired optogenetic stimulation of D2 NAc neuron fragments licking**
921 **microstructure but paradoxically promotes sucrose intake through its rewarding**
922 **effects.**

923 (**a**) Schematic representation of the brief access test with optogenetic stimulation. Mice
924 were subjected to varying delays (0, 50, and 100 ms) of 10 ms pulses of laser stimulation
925 entrained to licking during a 4 s reward period alongside a No-laser condition. (**b-d**)
926 PSTHs display the mean sucrose lick rates during the 4 s laser stimulation period for
927 $D2^{tdTomato}$ (**b**), $D2^{ChR2}$ (**c**), and $D2^{ArchT}$ (**d**). (**e-g**)Cumulative licks over the 20 min session
928 are shown for all conditions within the same group on the left y-axis, while total cumulative
929 licks (in purple) are shown on the right y-axis. (**h**) Inter-lick interval (ms) for each group
930 under different stimulation conditions. In the $D2^{ChR2}$ group, inter-lick intervals across all
931 conditions were significantly longer compared to the $D2^{tdTomato}$ group in the same
932 condition. In the $D2^{ArchT}$ group, the 0 ms delay condition showed a significantly shorter
933 inter-lick interval than the $D2^{tdTomato}$ group. (**i**) Number of licking bouts for each group
934 across conditions. The $D2^{ChR2}$ group exhibited significantly more bouts than the $D2^{tdTomato}$
935 group across all frequencies. (**j**) Bout size (licks) for each group. The $D2^{ChR2}$ group had
936 significantly smaller bout sizes than the $D2^{tdTomato}$ group in the No-laser, 0 ms delay, and
937 50 ms delay conditions. (**k**) Total trials initiated by each group under different stimulation
938 conditions. The activation of D2 neurons in the NAc led to significantly more trials initiated
939 in the $D2^{ChR2}$ group than in the $D2^{tdTomato}$ control group. Colored symbols: * significant
940 difference compared to $D2^{tdTomato}$ (control) at the same frequency, significant difference
941 compared to the No-laser condition within the same group. All significant differences had
942 $p < 0.05$. Data are mean ± SEM. The statistical analyses were conducted with one-way

ANOVA followed by Tukey-Kramer post hoc tests. $D2^{tdTomato}$ n=6, $D2^{ChR2}$ n=5, and $D2^{ArchT}$ n=5. Further analysis of the licking microstructure is presented in **Table 4**.

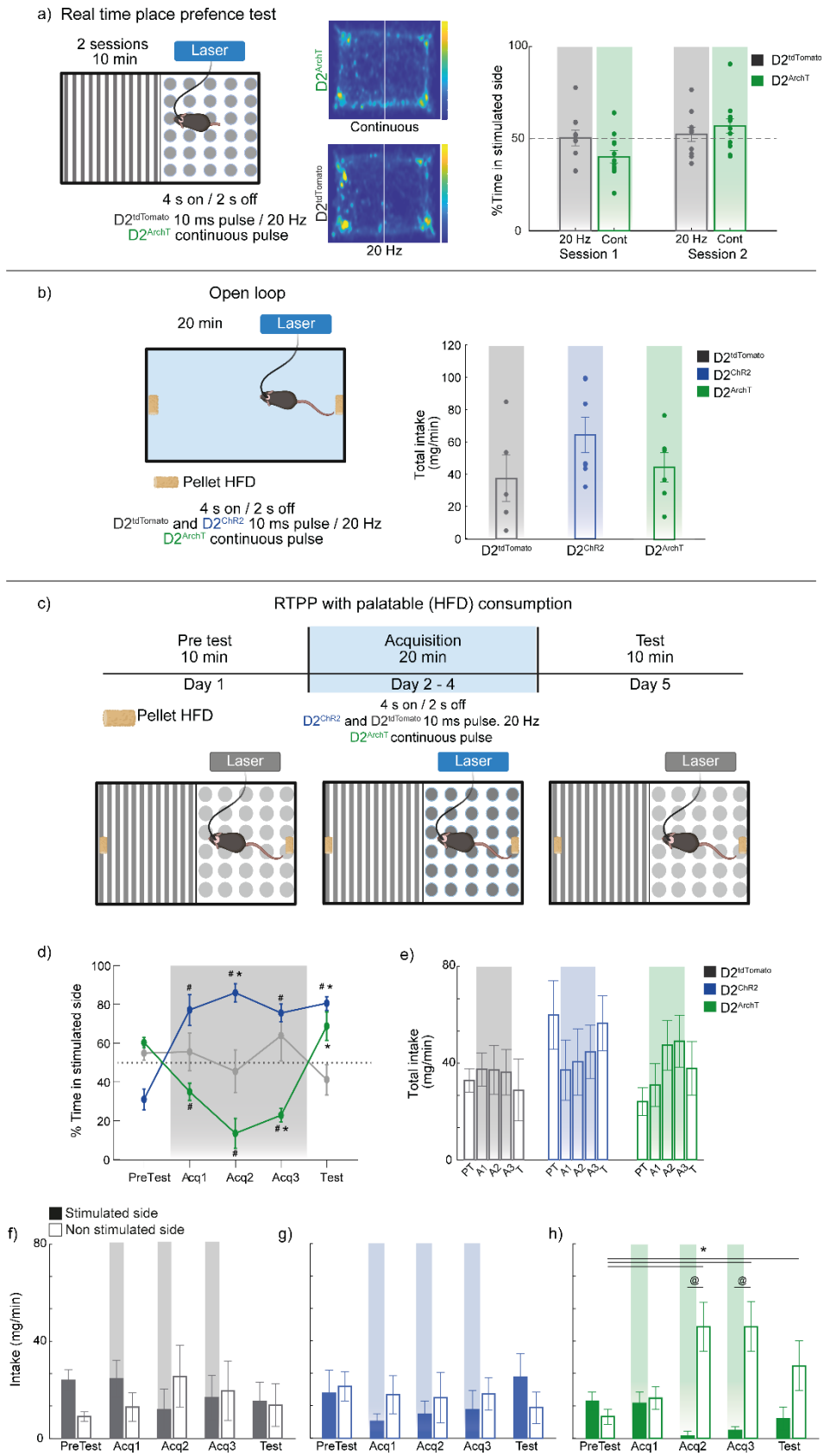


946 **Figure 5. Optogenetic activation of NAc D2 neurons at 20 Hz (but not 8 Hz) is**
947 **rewarding and consistently drives place preference.**

948 (**a**) Schematic representation of the Real-Time Place Preference (RTPP) task: Mice
949 explored a rectangular acrylic chamber divided into two visually distinct halves (one with
950 black/white stripes and the other with black/white circles). Upon entering the paired side,
951 mice received optogenetic stimulation for 4 s(10 ms pulse width at 20 Hz or 8 Hz for
952 $D2^{tdTomato}$ and $D2^{ChR2}$), followed by 2 s off, as long as mice remained on that side. Mice
953 performed two 10 min sessions, separated by approximately 5 h. The side paired with
954 stimulation was counterbalanced across subjects. (**b-e**) Heatmaps of mouse locations
955 during the task under different conditions: (**b**) $D2^{tdTomato}$ with 20 Hz stimulation, (**c**) $D2^{ChR2}$
956 with 20 Hz stimulation, (**d**) $D2^{tdTomato}$ with 8 Hz stimulation, and (**e**) $D2^{ChR2}$ with 8 Hz
957 stimulation (**f**) Percentage of time spent on the stimulated side during Session 1 and
958 Session 2. Mice expressing $D2^{ChR2}$ at 20 Hz spent significantly more time on the
959 stimulated side compared to $D2^{tdTomato}$ controls and the 8 Hz condition. No significant
960 preference was observed for 8 Hz groups. (**g**) Number of side crossings between the two
961 halves of the chamber. No significant differences were found across conditions. (**h**)
962 Velocity of the mice across conditions. The $D2^{ChR2}$ 20 Hz group had significantly higher
963 velocity on the non-stimulated side compared to the stimulated side and all other groups
964 and conditions on the non-stimulated side. (**i**) Total distance traveled during stimulation
965 sessions. The $D2^{ChR2}$ 20 Hz group traveled significantly more on the stimulated side than
966 $D2^{tdTomato}$ controls and other conditions. * significant difference compared to other groups
967 in the same session or side, # significant difference in the other side within the same
968 group. All significant differences had $p < 0.05$. Data are mean ± SEM. The statistical

969 analyses were conducted with one-way ANOVA followed by Tukey-Kramer post hoc

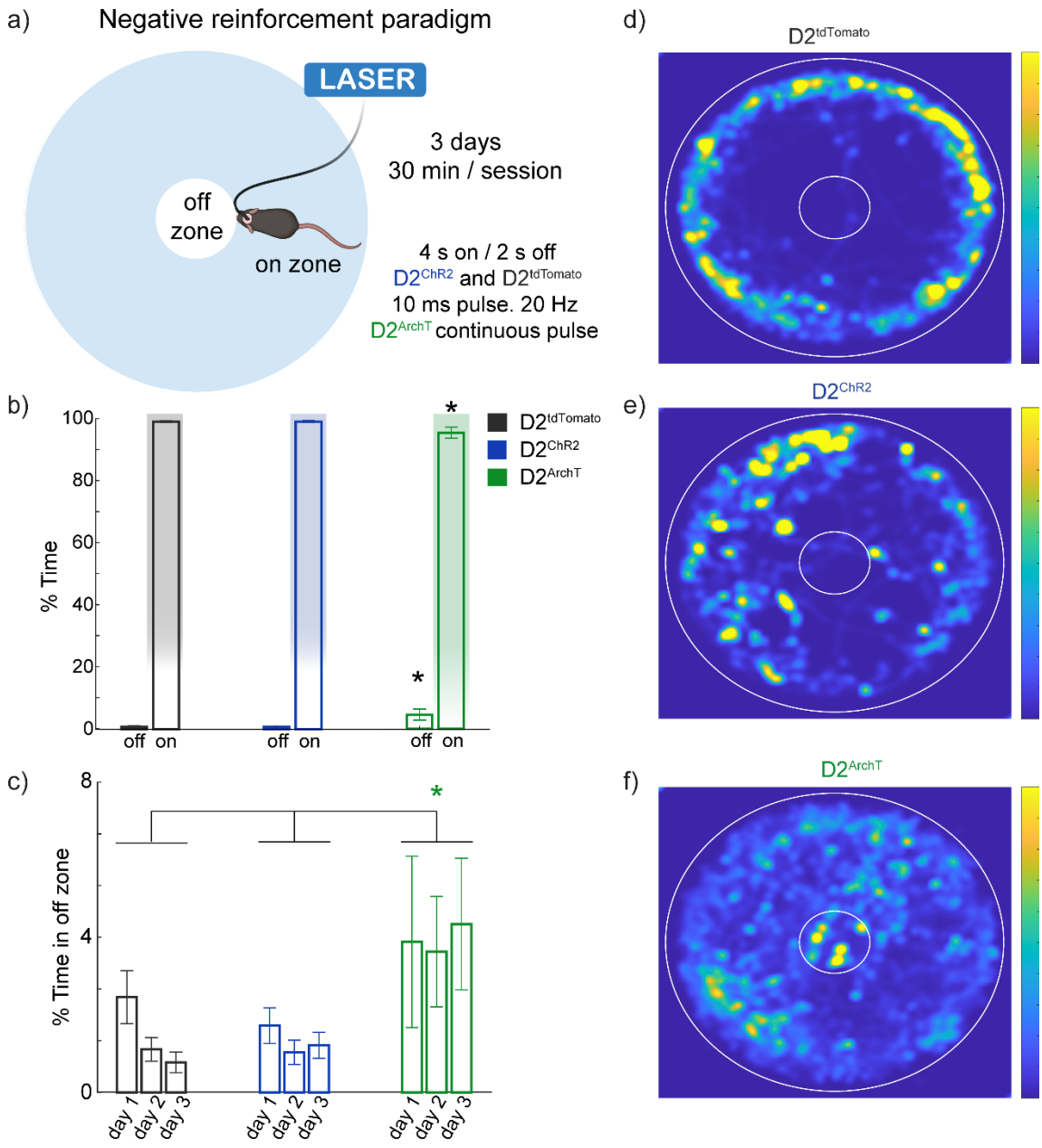
970 tests. $D2^{tdTomato}$ 8 Hz n=5, $D2^{ChR2}$ 8 Hz n=4, $D2^{tdTomato}$ 20 Hz n=9 and $D2^{ChR2}$ 20 Hz n=7.


a) Real time place prefence test
2 sessions
10 min
Laser
4 s on / 2 s off
D2tdTomato 10 ms pulse / 20 Hz
D2ArchT continuous pulse
D2ArchT
Continuous
D2tdTomato
20 Hz
% Time in stimulated side
D2tdTomato
D2ArchT
20 Hz Cont
Session 1
20 Hz Cont
Session 2
b)
Open loop
20 min
Laser
Pellet HFD
4 s on / 2 s off
D2tdTomato and D2ChR2 10 ms pulse / 20 Hz
D2ArchT continuous pulse
Total intake (mg/min)
D2tdTomato
D2ChR2
D2ArchT
c)
RTPP with palatable (HFD) consumption
Pre test
10 min
Acquisition
20 min
Test
10 min
Day 1
Day 2 - 4
Day 5
Pellet HFD
4 s on / 2 s off
D2ChR2 and D2tdTomato 10 ms pulse. 20 Hz
D2ArchT continuous pulse
Laser
Laser
Laser
d)
% Time in stimulated side
PreTest
Acq1
Acq2
Acq3
Test
e)
Total intake (mg/min)
D2tdTomato
D2ChR2
D2ArchT
PT
A1
A2
A3
T
Stimulated side
Non stimulated side
f)
g)
h)
Intake (mg/min)
PreTest
Acq1
Acq2
Acq3
Test

972 **Figure 6. Context-dependent effects of silencing NAc D2 neurons: inhibition only**
973 **alters HFD consumption based on environmental context**

974 (**a**) Left panel. Schematic representation of the RTPP task: Mice explored a rectangular
975 acrylic chamber divided into two visually distinct sides. Upon entering the laser paired
976 side, mice received optogenetic stimulation for 4 s (10 ms pulses at 20 Hz for $D2^{tdTomato}$
977 or continuous stimulation for $D2^{ArchT}$), followed by 2 s off. Mice experienced two 10 min
978 sessions, separated by approximately 5 h. The side paired with stimulation was
979 counterbalanced across subjects. Middle panels Heatmaps of mouse locations during the
980 task under different conditions: (middle-top) $D2^{ArchT}$ with continuous stimulation, (middle-
981 bottom) $D2^{tdTomato}$ with 20 Hz stimulation. Right panel percentage of time spent on the
982 stimulated side during session 1 and session 2. No significant preference was observed.
983 $D2^{tdTomato}$ 20 Hz n=9 (same data from **Fig 5**) and $D2^{ArchT}$ n=12. (**b**) Left panel. Schematic
984 of the open-loop paradigm used to assess the effects of optogenetic manipulation of D2-
985 NAc neurons on HFD intake. optogenetic stimulation was delivered in 4 s on / 2 s off
986 intervals, with continuous stimulation applied to the $D2^{ArchT}$ group, while a 20 Hz
987 frequency was used for the $D2^{ChR2}$ and $D2^{tdTomato}$ groups. Right panel. Total HFD intake
988 (mg/min) for $D2^{tdTomato}$ (control), $D2^{ChR2}$ (activation), and $D2^{ArchT}$ (inhibition) groups during
989 the 20 min session. $D2^{tdTomato}$ n=5, $D2^{ChR2}$ n=6, $D2^{ArchT}$ n=4. (**c**) Schematic representation
990 of the RTPP paradigm associated with palatable food intake. Mice were conditioned to
991 associate one side of the chamber with optogenetic stimulation (4 s on, 2 s off). $D2^{ChR2}$
992 and $D2^{tdTomato}$ groups received 10 ms pulses of 20 Hz stimulation, while the $D2^{ArchT}$ group
993 received continuous pulse stimulation. (**d**) Time course of the percentage of time spent
994 on the stimulation-paired side during pre-conditioning (PreTest), conditioning (Acq1,

995 Acq2, Acq3), and post-conditioning test (Test). Note that the $D2^{ChR2}$ group significantly
996 preferred the stimulated side during conditioning, consistently promoting place preference
997 in the test without stimulation. In contrast, silencing D2 neurons induces a tendency to
998 avoid the laser-paired side (**e**) Total HFD intake across conditioning days for all groups.
999 There is a tendency for reduced intake during the activation of NAc D2 neurons ($D2^{ChR2}$)
1000 and an opposite tendency for increased intake during inhibition ($D2^{ArchT}$). (**f-h**)
1001 Comparison of HFD intake on the stimulated versus non-stimulated sides for $D2^{tdTomato}$
1002 (**f**), $D2^{ChR2}$ (**g**), and $D2^{ArchT}$ (**h**) groups. The $D2^{ArchT}$ group showed a significant increase
1003 in HFD consumption on the non-stimulated side during the last two conditioning days
1004 compared to pre-conditioning. Gradient rectangles depict the condition or side paired with
1005 optogenetic stimulation. $D2^{tdTomato}$ n=7, $D2^{ChR2}$ n=7, $D2^{ArchT}$ n=5: * significant difference
1006 compared to $D2^{tdTomato}$ in the same session, # significant difference compared to the
1007 PreTest session within the same group, and @ significant difference between
1008 the stimulated and non-simulated side within the same group. All significant differences
1009 had $p < 0.05$. Data are mean ± SEM. The statistical analyses were conducted with one-
1010 way ANOVA followed by Tukey-Kramer post hoc tests.




1011

1012 **Figure 7. Silencing of NAc D2 neuron activity is hedonically negative and drives**

1013 **negative reinforcement**

1014 (**a**) Schematic illustration of the negative reinforcement paradigm. Mice were placed in a

1015 circular arena with distinct 'on' (optogenetic stimulation; 4 s on, 2 s off) and 'off' zones

1016 that turn off the stimulation. $D2^{ChR2}$ and $D2^{tdTomato}$ groups received pulsed stimulation in

1017 the ‘on’ zone, while D2ArchT received continuous stimulation. (**b**) Percentage of time
1018 spent in the ‘on’ and ‘off’ zones during the test for $D2^{tdTomato}$ (gray), $D2^{ChR2}$ (blue), and
1019 $D2^{ArchT}$ (green) groups. (**c**) Time course of the percentage of time spent in the initially ‘off’
1020 zone across the 3-day sessions. Mice expressing $D2^{ArchT}$ showed increased time spent
1021 in the ‘off’ zone on day 3, indicative of negative reinforcement behavior. (**d-f**)
1022 Representative heatmaps showing the spatial distribution of time spent by mice in the
1023 $D2^{tdTomato}$ (**d**), $D2^{ChR2}$ (**e**), and $D2^{ArchT}$ (**f**) groups during the post-conditioning test. *
1024 significant difference compared to other groups. All significant differences had $p < 0.05$.
1025 Data are mean ± SEM. The statistical analyses were conducted with one-way ANOVA
1026 followed by Tukey-Kramer post hoc tests. $D2^{tdTomato}$ n=6, $D2^{ChR2}$ n=4, $D2^{ArchT}$ n=4.
1027
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1033 # Tables

1034

1035 **Table 1. Analysis of licking microstructure for the two-bottle sucrose test (Fig 1).**

| | | $D2^{tdTomato}$ | | $D2^{ChR2}$ | | $D2^{ArchT}$ | |
|---|---|---|---|---|---|---|---|
| | | Water | Sucrose | Water | Sucrose | Water | Sucrose |
| Condition 1 Base line (no-laser) | Preference (%) | 21.69 ± 4.35 | 78.31 ± 4.35 | 11.96 ± 1.68 | 88.04 ± 1.68 | 14.53 ± 3.49 | 85.48 ± 3.49 |
| | Inter lick interval (ms) | 135.08 ± 3.35 | 130.32 ± 2.58 | 146.00 ± 5.99 | 134.43 ± 4.41 | 133.64 ± 2.90 | 128.94 ± 1.85 |
| | Lick rate (Hz) | 10.04 ± 0.47 | 9.64 ± 0.38 | 9.75 ± 0.39 | 9.50 ± 0.13 | 10.15 ± 0.52 | 9.40 ± 0.27 |
| | Bout size (Licks) | 14.10 ± 3.53 | 23.18 ± 5.33 | 9.89 ± 1.61 | 19.41 ± 2.03 | 12.96 ± 2.46 | 28.38 ± 4.57 |
| | Bout duration (s) | 1.71 ± 0.44 | 2.88 ± 0.69 | 1.23 ± 0.21 | 2.40 ± 0.26 | 1.60 ± 0.33 | 3.55 ± 0.61 |
| | Inter bout interval (s) | 82.49 ± 11.96 | 39.96 ± 3.88 | 101.50 ± 25.40 | 22.18 ± 2.45 | 101.34 ± 24.55 | 29.76 ± 2.88 |
| | Number of bouts | 14.31 ± 2.09 | 35.54 ± 3.52 | 17.86 ± 2.54 | 70.14 ± 7.58 | 14.94 ± 2.77 | 48.82 ± 6.89 |
| | Total licks | 182.92 ± 39.79 | 714.00 ± 119.42 | 152.41 ± 24.99 | 1140.09 ± 94.84 | 154.24 ± 34.42 | 1198.82 ± 154.07 |
| | Total licks sum | 896.92 ± 132.45 | | 1292.50 ± 101.87 | | 1353.06 ± 150.57 | |
| Condition 2 Laser sucrose | Preference (%) | 16.32 ± 3.70 | 83.68 ± 3.70 | **#*40.43 ± 4.28** | **#*59.57 ± 4.28** | 20.84 ± 4.48 | 79.16 ± 4.48 |
| | Inter lick interval (ms) | 137.22 ± 3.40 | 130.23 ± 1.83 | 136.46 ± 2.75 | **#*160.19 ± 4.25** | 137.23 ± 4.54 | 128.70 ± 3.65 |
| | Lick rate (Hz) | 9.59 ± 0.63 | 9.08 ± 0.25 | 9.17 ± 0.29 | **#*9.86 ± 0.23** | **#8.86 ± 0.36** | 9.57 ± 0.26 |
| | Bout size (Licks) | 20.14 ± 4.61 | 32.34 ± 3.65 | **#22.68 ± 2.05** | **#*5.66 ± 0.57** | 29.22 ± 7.40 | 25.74 ± 4.02 |
| | Bout duration (s) | 2.59 ± 0.61 | 4.09 ± 0.47 | **#2.93 ± 0.29** | **#*0.72 ± 0.08** | 3.65 ± 0.95 | 3.20 ± 0.53 |
| | Inter bout interval (s) | 172.61 ± 85.84 | 45.11 ± 5.54 | 52.88 ± 5.41 | **#*14.78 ± 1.66** | 64.72 ± 24.94 | **#28.36 ± 3.43** |
| | Number of bouts | 9.00 ± 1.68 | 32.78 ± 4.03 | **#18.25 ± 1.66** | **#*121.21 ± 14.65** | 13.91 ± 2.80 | **#64.04 ± 10.03** |
| | Total licks | 172.61 ± 44.05 | 939.39 ± 123.44 | **#*414.42 ± 45.60** | **#659.25 ± 91.50** | 243.65 ± 45.51 | 1298.61 ± 166.53 |
| | Total licks sum | 1112.00 ± 122.96 | | **#*1073.67 ± 103.77** | | **#*1542.26 ± 159.17** | |
| Condition 3 Laser water | Preference (%) | 20.63 ± 5.18 | 79.37 ± 5.18 | 13.34 ± 3.34 | 86.66 ± 3.34 | **9.57 ± 1.80** | **90.43 ± 1.80** |
| | Inter lick interval (ms) | 145.94 ± 12.51 | 132.34 ± 2.61 | **#166.96 ± 7.05** | 134.50 ± 1.89 | 139.97 ± 10.09 | 128.62 ± 2.22 |
| | Lick rate (Hz) | 10.50 ± 0.72 | 9.09 ± 0.35 | 9.38 ± 0.38 | **#8.71 ± 0.21** | 9.93 ± 0.57 | 9.71 ± 0.28 |
| | Bout size (Licks) | 17.44 ± 4.60 | 31.50 ± 5.04 | **#*5.15 ± 1.49** | **#30.51 ± 3.55** | 14.14 ± 6.83 | 22.69 ± 3.98 |
| | Bout duration (s) | 2.18 ± 0.59 | 4.02 ± 0.69 | **#0.65 ± 0.23** | **#3.93 ± 0.44** | 1.70 ± 0.87 | 2.76 ± 0.50 |
| | Inter bout interval (s) | 168.50 ± 42.56 | 61.76 ± 5.59 | **#*24.90 ± 2.91** | **#28.28 ± 4.61** | **78.05 ± 12.98** | **#*20.22 ± 2.93** |
| | Number of bouts | 9.42 ± 1.53 | 23.92 ± 2.80 | **#*58.38 ± 14.21** | **#61.69 ± 8.02** | 17.82 ± 4.70 | **#78.82 ± 17.14** |
| | Total licks | 161.00 ± 41.41 | 647.25 ± 93.14 | 245.38 ± 66.20 | **#*1588.12 ± 137.07** | 132.73 ± 24.25 | **#1475.27 ± 223.91** |
| | Total licks sum | 808.25 ± 89.56 | | **#*1833.50 ± 142.29** | | **#*1608.00 ± 231.36** | |

1036

1037 Bold font indicates statistically significant differences ($p<0.05$):

1038 *Significant difference compared to the $D2^{tdTomato}$ (control group) in the same condition.

1039 **#**Significant difference compared to Baseline (condition 1) within the same group.

1040 **Table 2. Analysis of licking microstructure for sucrose freely licking with**

1041 **optogenetic frequency scan test (Fig 2).**

| | | | $D2^{tdTomato}$ | $D2^{ChR2}$ | $D2^{ArchT}$ |
|---|---|---|---|---|---|
| Stimualtion Frequencies | No-laser | Inter lick interval (ms) | 159.75 ± 7.40 | **#168.31 ± 5.45** | **#162.87 ± 5.26** |
| | | Lick rate (Hz) | 8.06 ± 0.35 | 8.28 ± 0.29 | 7.93 ± 0.16 |
| | | Bout size (Licks) | 5.36 ± 0.26 | 4.71 ± 0.23 | 5.37 ± 0.17 |
| | | Bout duration (s) | 0.68 ± 0.02 | 0.61 ± 0.03 | 0.69 ± 0.01 |
| | | Inter bout interval (s) | 154.56 ± 21.92 | 194.52 ± 20.23 | 145.27 ± 13.77 |
| | | Number of bouts | 7.50 ± 0.96 | 6.63 ± 0.88 | 9.00 ± 1.01 |
| | | Total licks | 41.00 ± 6.44 | 32.81 ± 5.71 | 52.48 ± 7.32 |
| | 5 Hz | Inter lick interval (ms) | 176.21 ± 16.76 | **#202.76 ± 8.79** | **#165.96 ± 5.21** |
| | | Lick rate (Hz) | 7.62 ± 0.37 | 7.70 ± 0.34 | 7.81 ± 0.20 |
| | | Bout size (Licks) | 4.77 ± 0.28 | **#*3.64 ± 0.23** | 5.19 ± 0.16 |
| | | Bout duration (s) | 0.62 ± 0.03 | **#*0.50 ± 0.03** | 0.67 ± 0.01 |
| | | Inter bout interval (s) | 148.77 ± 23.21 | 211.43 ± 25.26 | 157.95 ± 20.53 |
| | | Number of bouts | 7.67 ± 0.99 | 6.67 ± 0.85 | 8.73 ± 1.01 |
| | | Total licks | 37.92 ± 5.97 | 25.85 ± 4.78 | 48.64 ± 6.99 |
| | 8 Hz | Inter lick interval (ms) | 168.78 ± 9.86 | **#*234.59 ± 11.70** | **#171.09 ± 6.46** |
| | | Lick rate (Hz) | 8.15 ± 0.44 | #7.11 ± 0.33 | 7.78 ± 0.20 |
| | | Bout size (Licks) | 4.63 ± 0.32 | **#*2.81 ± 0.19** | **#4.75 ± 0.19** |
| | | Bout duration (s) | 0.58 ± 0.03 | **#*0.39 ± 0.03** | **#0.61 ± 0.02** |
| | | Inter bout interval (s) | 122.57 ± 18.60 | 204.67 ± 24.27 | 146.39 ± 19.22 |
| | | Number of bouts | 7.75 ± 1.05 | 5.89 ± 0.77 | 8.64 ± 1.01 |
| | | Total licks | 38.33 ± 6.85 | **#18.00 ± 3.34** | 45.18 ± 6.61 |
| | 15 Hz | Inter lick interval (ms) | 156.65 ± 7.71 | **#*221.38 ± 12.27** | **#171.81 ± 5.07** |
| | | Lick rate (Hz) | 8.36 ± 0.36 | 7.53 ± 0.41 | 7.70 ± 0.20 |
| | | Bout size (Licks) | 4.97 ± 0.36 | **#*2.63 ± 0.28** | 4.89 ± 0.19 |
| | | Bout duration (s) | 0.60 ± 0.05 | **#*0.32 ± 0.05** | **#0.64 ± 0.02** |
| | | Inter bout interval (s) | 121.84 ± 16.57 | 173.98 ± 19.82 | 181.09 ± 37.38 |
| | | Number of bouts | 7.92 ± 1.01 | **5.19 ± 0.74** | 8.67 ± 1.00 |
| | | Total licks | 40.83 ± 6.86 | **#15.44 ± 3.30** | 46.64 ± 7.08 |
| | 20 Hz | Inter lick interval (ms) | 168.95 ± 9.95 | **#*214.47 ± 10.03** | **#166.83 ± 5.49** |
| | | Lick rate (Hz) | 7.92 ± 0.41 | 8.24 ± 0.45 | 8.20 ± 0.22 |
| | | Bout size (Licks) | 4.78 ± 0.22 | **#*2.43 ± 0.27** | **#4.58 ± 0.19** |
| | | Bout duration (s) | 0.62 ± 0.03 | **#*0.28 ± 0.05** | **#0.57 ± 0.02** |
| | | Inter bout interval (s) | 140.67 ± 25.52 | 229.92 ± 28.06 | 159.46 ± 18.53 |
| | | Number of bouts | 7.58 ± 1.00 | 4.96 ± 0.77 | 8.70 ± 0.93 |
| | | Total licks | 37.08 ± 5.61 | **#*13.00 ± 2.56** | 44.15 ± 6.46 |

1042

1043 Bold font indicates statistically significant differences ($p<0.05$):

1044 *****Significant difference compared to the $D2^{tdTomato}$ (control group) at the same frequency.

1045 **#**Significant difference compared to the Control (no laser) frequency within the same

1046 group.

1047

1048 **Table 3. Analysis of licking microstructure for sucrose BATT with frequency scan**

1049 **test (Fig 3).**

| Stimulation pattern | | $D2^{tdTomato}$ | $D2^{ChR2}$ | $D2^{ArchT}$ |
|---|---|---|---|---|
| No-laser | Inter lick interval (ms) | 171.05 ± 7.13 | 168.30 ± 8.88 | ***150.41 ± 4.78** |
| | Lick rate (Hz) | 4.02 ± 0.26 | 4.10 ± 0.47 | ***5.04 ± 0.32** |
| | Bout size (licks) | 15.73 ± 1.04 | 16.00 ± 1.97 | ***20.00 ± 1.28** |
| | Bout duration (s) | 2.40 ± 0.12 | 2.33 ± 0.25 | 2.82 ± 0.17 |
| | Inter bout interval (s) | 129.94 ± 11.21 | ***83.82 ± 9.69** | 178.04 ± 33.50 |
| | Number of bouts | 8.00 ± 1.02 | ***16.10 ± 2.14** | 6.80 ± 0.62 |
| | Total licks | 124.77 ± 22.91 | ***214.71 ± 23.36** | 122.36 ± 7.36 |
| Lick paired | Inter lick interval (ms) | 174.78 ± 9.90 | 190.00 ± 8.97 | ***148.58 ± 3.94** |
| | Lick rate (Hz) | 4.13 ± 0.38 | 3.54 ± 0.32 | 4.55 ± 0.29 |
| | Bout size (licks) | 16.04 ± 1.56 | 13.53 ± 1.43 | 18.07 ± 1.14 |
| | Bout duration (s) | 2.37 ± 0.17 | 2.24 ± 0.22 | 2.49 ± 0.15 |
| | Inter bout interval (s) | 152.61 ± 18.14 | ***79.15 ± 9.33** | 150.76 ± 16.10 |
| | Number of bouts | 8.68 ± 1.29 | ***16.14 ± 1.89** | 7.16 ± 0.52 |
| | Total licks | 127.09 ± 24.92 | 187.14 ± 18.81 | 121.16 ± 7.88 |
| 8 Hz | Inter lick interval (ms) | 172.28 ± 8.50 | 187.64 ± 9.91 | ***143.06 ± 3.34** |
| | Lick rate (Hz) | 4.00 ± 0.37 | **@#*2.54 ± 0.29** | ***5.29 ± 0.31** |
| | Bout size (licks) | 15.13 ± 1.62 | **#*9.89 ± 1.18** | ***20.88 ± 1.26** |
| | Bout duration (s) | 2.24 ± 0.20 | **@#*1.55 ± 0.17** | ***2.81 ± 0.16** |
| | Inter bout interval (s) | 129.70 ± 14.18 | ***68.95 ± 8.01** | 159.22 ± 15.26 |
| | Number of bouts | 8.82 ± 1.22 | ***18.38 ± 2.15** | 6.48 ± 0.58 |
| | Total licks | 119.91 ± 21.86 | **#150.19 ± 15.43** | 125.20 ± 8.88 |
| 20 Hz | Inter lick interval (ms) | 171.02 ± 8.56 | 180.40 ± 9.14 | ***144.91 ± 4.74** |
| | Lick rate (Hz) | 4.16 ± 0.36 | **#*2.81 ± 0.40** | 5.13 ± 0.40 |
| | Bout size (licks) | 16.44 ± 1.46 | **@#*8.64 ± 1.66** | 18.80 ± 1.64 |
| | Bout duration (s) | 2.46 ± 0.16 | **@#*1.30 ± 0.28** | 2.51 ± 0.19 |
| | Inter bout interval (s) | 142.75 ± 13.16 | ***87.00 ± 9.26** | 175.73 ± 30.39 |
| | Number of bouts | 7.36 ± 0.88 | ***14.57 ± 1.58** | 6.72 ± 0.56 |
| | Total licks | 119.64 ± 22.17 | **!@#97.14 ± 14.57** | 121.04 ± 10.41 |

1050

1051 Bold font indicates statistically significant differences ($p < 0.05$):

1052 ***** Significant difference compared to the $D2^{tdTomato}$ (control group) at the same frequency.

1053 **#** Significant difference compared to the Control (no laser) frequency within the same

1054 group.

1055 **@** Significant difference between the Lick-paired condition and the 8 Hz or 20 Hz

1056 conditions within the same group

1057 **!** Significant difference between the 8 Hz and 20 Hz conditions within the same group

1058

1059 **Table 4. Analysis of licking microstructure analysis of licking for the sucrose BATT**

1060 **with varying optogenetic stimulation delay (Fig 4).**

| Stimulation pattern | | $D2^{tdTomato}$ | $D2^{ChR2}$ | $D2^{ArchT}$ |
|---|---|---|---|---|
| No-laser | Inter lick interval (ms) | 150.95 ± 5.76 | ***179.48 ± 8.71** | 138.06 ± 4.97 |
| | Lick rate (Hz) | 5.04 ± 0.36 | ***3.48 ± 0.57** | 5.26 ± 0.45 |
| | Bout size (licks) | 19.89 ± 1.50 | ***13.85 ± 2.30** | 20.75 ± 1.80 |
| | Bout duration (s) | 2.80 ± 0.22 | 2.18 ± 0.36 | 2.74 ± 0.28 |
| | Inter bout interval (s) | 191.64 ± 21.62 | ***84.71 ± 11.81** | 200.87 ± 38.04 |
| | Number of bouts | 5.60 ± 0.72 | ***15.70 ± 2.97** | 5.50 ± 1.09 |
| | Total licks | 110.07 ± 19.52 | 181.00 ± 36.21 | 111.12 ± 21.37 |
| 0 ms delay (lick paired) | Inter lick interval (ms) | 156.21 ± 6.95 | ***206.89 ± 13.27** | 134.84 ± 5.75 |
| | Lick rate (Hz) | 4.76 ± 0.44 | ***2.68 ± 0.46** | 5.23 ± 0.64 |
| | Bout size (licks) | 18.74 ± 1.76 | ***10.53 ± 1.85** | 20.78 ± 2.55 |
| | Bout duration (s) | 2.65 ± 0.19 | ***1.81 ± 0.27** | 2.58 ± 0.26 |
| | Inter bout interval (s) | 206.87 ± 27.54 | ***79.25 ± 12.82** | 174.32 ± 20.18 |
| | Number of bouts | 6.20 ± 0.94 | ***18.20 ± 3.54** | 4.88 ± 0.74 |
| | Total licks | 110.73 ± 19.11 | 159.10 ± 32.62 | 96.75 ± 14.28 |
| 50 ms delay | Inter lick interval (ms) | 151.28 ± 8.05 | ***190.58 ± 8.62** | 149.19 ± 5.76 |
| | Lick rate (Hz) | 4.57 ± 0.43 | ***2.88 ± 0.45** | 5.12 ± 0.75 |
| | Bout size (licks) | 18.16 ± 1.74 | ***11.36 ± 1.83** | 20.22 ± 3.03 |
| | Bout duration (s) | 2.48 ± 0.21 | 1.87 ± 0.29 | 2.76 ± 0.34 |
| | Inter bout interval (s) | 209.14 ± 29.89 | ***82.81 ± 12.84** | 171.57 ± 35.85 |
| | Number of bouts | 6.27 ± 0.75 | ***18.00 ± 3.81** | 5.88 ± 0.79 |
| | Total licks | 105.87 ± 16.64 | 167.70 ± 34.42 | 114.12 ± 19.19 |
| 100ms delay | Inter lick interval (ms) | 147.26 ± 5.72 | ***188.82 ± 7.84** | 139.84 ± 7.25 |
| | Lick rate (Hz) | 4.82 ± 0.44 | ***3.32 ± 0.57** | 6.39 ± 0.71 |
| | Bout size (licks) | 18.87 ± 1.83 | 13.26 ± 2.30 | 25.14 ± 2.84 |
| | Bout duration (s) | 2.53 ± 0.23 | 2.21 ± 0.38 | 3.25 ± 0.28 |
| | Inter bout interval (s) | 212.17 ± 21.39 | ***84.43 ± 13.25** | 148.41 ± 20.66 |
| | Number of bouts | 5.47 ± 0.72 | ***17.00 ± 3.47** | 4.88 ± 0.52 |
| | Total licks | 97.00 ± 14.35 | ***178.50 ± 32.34** | 119.88 ± 17.51 |

1061

1062 Bold indicates statistically significant differences ($p < 0.05$):

1063 ***** Significant difference compared to the $D2^{tdTomato}$ (control group) in the same condition

1064 (delay time).