

1 **Opposing roles of dopamine receptor D1- and D2-expressing neurons**
2 **in the anteromedial olfactory tubercle in acquisition of place preference**
3 **in mice**

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26

27 **Abstract**

28 Olfaction induces adaptive motivated behaviors. Odors associated with food induce attractive
29 behavior, whereas those associated with dangers induce aversive behavior. We previously reported
30 that learned odor-induced attractive and aversive behaviors accompany activation of the olfactory
31 tubercle (OT) in a domain- and cell type-specific manner. Odor cues associated with a sugar reward
32 induced attractive behavior and c-fos expression in the dopamine receptor D1-expressing neurons
33 (D1 neurons) in the anteromedial OT. In contrast, odor cues associated with electrical shock induced
34 aversive behavior and c-fos expression in the D2 neurons in the anteromedial OT, as well as the D1
35 neurons in the lateral OT. Here, we investigated whether the D1 and D2 neurons in the anteromedial
36 OT play distinct roles in attractive or aversive behaviors, using optogenetic stimulation and real-time
37 place preference (RTPP) tests. Mice expressing ChETA (ChR2/E123T)-EYFP in the D1 neurons in
38 the anteromedial OT spent a longer time in the photo-stimulation side of the place preference
39 chamber than the control mice expressing EYFP. On the other hand, upon optogenetic stimulation of
40 the D2 neurons in the anteromedial OT, the mice spent a shorter time in the photo-stimulation side
41 than the control mice. Local neural activation in the anteromedial OT during the RTPP tests was
42 confirmed by c-fos mRNA expression. These results suggest that the D1 and D2 neurons in the
43 anteromedial OT play distinct roles in attractive and aversive behaviors, respectively.

44

45 **1 Introduction**

46 Odor sensation elicits various motivations, which enable adaptive behavioral responses such as
47 obtaining food rewards or avoiding potential dangers (Doty, 1986). Although some odorants elicit
48 innate motivated behaviors in mice, such as fear responses to predator odors (Kobayakawa et al.,
49 2007; Saito et al., 2017) or attractive responses to social odors (Inokuchi et al., 2017), animals can
50 acquire appropriate behaviors to odor cues according to their experience, through odor-reward or
51 odor-danger associative learning. However, the neural circuit mechanisms engaged in these odor-
52 induced adaptive behaviors are still unclear.

53 Recent studies have revealed the importance of the olfactory tubercle (OT) in the odor-
54 induced motivated behaviors (DiBenedictis et al., 2015; Gadziola et al., 2015; Yamaguchi, 2017;
55 Zhang et al., 2017a; Murofushi et al., 2018). The OT is a part of the olfactory cortex that receives
56 olfactory inputs directly from the olfactory bulb as well as indirectly from other parts of the olfactory
57 cortex and the orbitofrontal cortex (Shepherd, 2004; Zhang et al., 2017b). The OT is also a part of the
58 ventral striatum, in addition to the nucleus accumbens (NAc), that receives massive dopaminergic
59 inputs from the ventral tegmental area (Ikemoto, 2007; Zhang et al., 2017b; Poulin et al., 2018). The
60 OT is composed of three major types of neurons: medium spiny neurons, dwarf cells, and granule
61 cells (Millhouse and Heimer, 1984; Xiong and Wesson, 2016). The medium spiny neurons are
62 distributed in the whole OT, forming the layer II (dense cell layer) of the cortex-like region
63 (Millhouse and Heimer, 1984). A majority of the medium spiny neurons in the OT as well as the
64 NAc and dorsal striatum express either dopamine receptor D1 or D2 (Yung et al., 1995; Murata et al.,
65 2015). Dwarf cells are clustered in the lateral part of the OT, forming the cap region, which is
66 interspersed throughout the antero-posterior axis (Hosoya and Hirata, 1974; Murata et al., 2015). The
67 dwarf cells are considered a smaller type of the medium spiny neurons, and express D1 but not D2
68 (Murata et al., 2015). Granule cells are clustered through the anteromedial surface to the central deep
69 part of the OT, forming the Islands of Calleja, which is presumably a continuous structure (Fallon et
70 al., 1978; de Vente et al., 2001). The granule cells weakly express D1, and do not express D2
71 (Murata et al., 2015). In addition to these three types of neurons in the striatal component, the OT
72 contains the ventral pallidal component and axon bundles that project from the striato-pallidal
73 structure to other brain areas, forming the medial forebrain bundle (Heimer, 1978).

74 In our previous study, we divided the OT into domains, using the cap and Islands of Calleja
75 as a landmark, and mapped *c-fos* expression when mice showed learned odor-induced attractive or
76 aversive behaviors (Murata et al., 2015). Odor cues associated with a sugar reward induced attractive
77 behavior and *c-fos* expression in the D1-expressing neurons (D1 neurons) in the cortex-like region of
78 the anteromedial domain, which is covered by the superficially located Islands of Calleja. In contrast,
79 odor cues associated with electrical shock induced aversive behavior and *c-fos* expression in the D2-
80 expressing neurons (D2 neurons) in the cortex-like region of the anteromedial domain, as well as D1
81 neurons in the cap and cortex-like regions of the lateral domain, which is surrounded by the cap
82 region. These results raise the possibility that the D1 and D2 neurons in the anteromedial OT play
83 opposing roles in odor-guided motivated behaviors. Consistent with this idea, the D1 and D2 neurons
84 in the NAc have distinct roles in attractive and aversive learning (Hikida et al., 2010). Here, we
85 investigated whether activation of the D1 and D2 neurons induces attractive and aversive behaviors,
86 respectively, by combining optogenetic stimulation and real-time place preference (RTPP) tests
87 (Zhang et al., 2017a).

88

89 2 Materials and Methods

90 Animals

91 All experiments were conducted in accordance with the Guidelines for Animal Experimentation in
92 Neuroscience of the Japan Neuroscience Society, and were approved by the Experimental Animal
93 Research Committee of University of Fukui. The D1-Cre and D2-Cre mice used were heterozygotes
94 and bred from D1-Cre (the Mutant Mouse Resource & Research Centers, STOCK Tg(Drd1a-
95 cre)FK150Gsat/Mmucd, stock number: 029178-UCD) (Gong et al., 2003; Gong et al., 2007) and D2-
96 Cre (the Mutant Mouse Resource & Research Centers, B6.FVB(Cg)-Tg(Drd2-
97 cre)ER44Gsat/Mmucd, stock number: 032108-UCD) (Gong et al., 2003; Gong et al., 2007) by
98 mating the heterozygote transgenic mice with wild type C57BL/6J mice (Japan SLC). All mice were
99 housed with their littermates until the surgery and then individually housed with a 12/12-h light/dark
100 cycle. Food and water were freely available.

101 Virus preparation

102 We used a Cre-dependent adeno-associated virus (AAV) vector encoding enhanced yellow
103 fluorescent protein (EYFP) or ChETA(ChR2/E123T)-EYFP for cell type-specific gene delivery. We
104 obtained AAV5-EF1a-DIO-EYFP from the UNC vector core at a titer of 3.5×10^{12} genome
105 copies/mL; AAV2-EF1a-DIO-ChETA-EYFP was packaged and concentrated to a titer of 1.6×10^{12}
106 genome copies/mL, as previously reported (Kobayashi et al., 2016), using the Addgene (Cambridge,
107 MA, USA) plasmid, pAAV-EF1a-DIO ChETA-EYFP (gift from Karl Deisseroth, # 26968 (Gunaydin
108 et al., 2010)).

109 Stereotaxic surgery

110 Stereotaxic surgeries were performed on mice aged 10–16 weeks. Mice were anesthetized with a
111 mixture of three anesthetics (medetomidine, midazolam, and butorphanol) (Nakamura et al., 2017),
112 and then placed in a stereotaxic apparatus (Narishige, SR-5M). The skull above the targeted areas
113 was thinned using a dental drill and removed carefully. Injections were administered using a syringe
114 pump (WPI, UltraMicroPump III) connected to a Hamilton syringe (Hamilton, RN-1701), and a
115 mounted glass micropipette with a tip diameter of 50 μ m connected by an adaptor (Hamilton, 55750-
116 01).

117 We ipsilaterally injected 300 nL AAV5-EF1a-DIO-EYFP for confirmation of cell type-
118 specific expression (Fig. 1C–D) and as a control for optogenetic stimulation, or AAV2-EF1a-DIO-
119 ChETA-EYFP into the anteromedial OT of D1-Cre or D2-Cre mice using the following coordinates:
120 anterior-posterior, +1.5 mm; medial-lateral, 0.7 mm from bregma; and dorso-ventral, 4.35 mm from
121 the brain surface. Two to 3 weeks later, the mice were ipsilaterally implanted with a chronic optical
122 fiber (numerical aperture = 0.39, 200- μ m diameter; Thorlabs, CFMC12U) targeted to the
123 anteromedial OT with the same coordinates described above. One to 2 weeks after fiber implantation,
124 the following behavioral tests were conducted.

125 Optogenetic stimulation and RTPP tests

126 For optogenetic stimulation, the implanted optic fiber was connected to a blue light laser via patch
127 cords with a fiber-optic rotary joint (Thorlabs, RJPSF2). All photo-stimulation experiments used 5-
128 ms, 5–7-mW, 473-nm light pulses at 20 Hz via a solid-state laser for light delivery (Ultra Laser,
129 CST-L-473-50-OEM) triggered by a stimulator (Bio Research Center, STO2).

130 After being connected to the blue light laser, the mice were placed in a place preference
131 chamber (30 [width] × 30 [depth] × 25 [height] cm) equipped with vertical or horizontal striped wall,
132 as shown in Fig. 1B, for 20 min. The non-stimulation control side was as assigned at the start of the
133 experiment. Laser stimulation at 20 Hz was constantly delivered when the mice crossed to the
134 stimulation side, and stopped when they returned back to the initial non-stimulation side. All
135 behavioral tests were recorded using a USB digital video camera (Logicool c920r), and offline
136 analyses of the time spent in each chamber and tracking data were performed using a videotracking
137 software (Panlab, SMART 3.0); 30 min after the end of the RTPP tests, mice were deeply
138 anesthetized by intraperitoneal injection of sodium pentobarbital, and then fixed for histochemical
139 analysis.

140 **Histochemistry**

141 Mice were transcardially perfused with phosphate-buffered saline (PBS), followed by 4%
142 paraformaldehyde (PFA). After cryoprotection with sucrose solution, the brain was frozen and sliced
143 into coronal sections with a thickness of 20 μ m. The sections were rinsed in PBS and 0.1 M
144 phosphate buffer, mounted on glass slides using a paint brush, dried overnight in a vacuum
145 desiccator, and then stored at 4 °C until histochemistry.

146 To confirm cell type-specific EYFP expression, we performed double fluorescent
147 immunolabelling for EYFP and mRNAs of D1 or D2 as follows. Digoxigenin (DIG)-labeled RNA
148 probes were prepared using an *in vitro* transcription kit (Roche) according to the manufacturer's
149 protocol with a plasmid kindly provided by Dr. Kazuto Kobayashi (Sano et al., 2003). The dried
150 sections were fixed in 4% PFA, digested using proteinase K (10 μ g/mL) for 30 min, and post-fixed in
151 4% PFA. After prehybridization, the sections were incubated overnight at 65 °C with DIG-labelled
152 RNA probes. After stringent washing, the sections were incubated in 1% blocking buffer (Roche,
153 11096176001) for 1 h. Primary antibody against EYFP (1:1000; Medical & Biological Laboratories)
154 and an anti-DIG antibody conjugated with alkaline phosphatase (1:500, Roche) were included in the
155 incubation mixture. The sections were washed three times in TNT (0.1 M Tris-HCl [pH 7.5], 0.15 M
156 NaCl, 0.1% Tween 20) and incubated with an Alexa Fluor 488-conjugated secondary antibody
157 (1:400; Jackson ImmunoResearch Labs) for 2 h. After three washes in TNT and one wash in Tris
158 saline (0.1 M Tris-HCl [pH 8.0], 0.1 M NaCl, 50 mM MgCl₂), alkaline phosphatase activity was
159 detected using the HNPP Fluorescence Detection Set (Roche, 11758888001) according to the
160 manufacturer's instructions. The sections were incubated with the substrate three times for 30 min
161 each, and the reaction was stopped by washing the sections in PBS. The sections were then
162 counterstained with 4',6-diamidino-2-phenylindole diluted in PBS (2 μ g/mL) for 5 min. After
163 washing in PBS, the sections were mounted in PermaFluor (Thermo Fisher Scientific).

164 For *c-fos* mRNA detection, we performed *in situ* hybridization using DIG-labeled antisense
165 RNA probes. The RNA probe was prepared using an *in vitro* transcription kit (Roche) according to
166 the manufacturer's protocol with a plasmid kindly provided by Dr. Hirohide Takebayashi (Bepari et
167 al., 2012). Hybridization and washing were performed as described above. Subsequently, the sections
168 were blocked with 10% normal sheep serum, 1% bovine serum albumin, and 0.1% Triton X-100 in
169 PBS. Subsequently, the sections were incubated overnight at 4 °C with alkaline phosphatase-
170 conjugated anti-DIG antibody (1:1000, Roche). The sections were washed in TNT, followed by
171 alkaline phosphatase buffer (100 mM NaCl, 100 mM Tris-HCl [pH 9.5], 50 mM MgCl₂, 0.1% Tween
172 20, 5 mM levamisole). The sections were treated overnight with nitro-blue tetrazolium/5-bromo-4-
173 chloro-3'-indolylphosphate (Roche) mixture at room temperature in a dark room for color
174 development. Then, they were rinsed in PBS and mounted in PermaFluor (Thermo Fisher Scientific).

175 **Microscopy and image analysis**

176 Sections were examined using a confocal laser microscope (Olympus, FV1200) and a bright field
177 virtual slide system (Hamamatsu Photonics, NanoZoomer). To quantify density of c-fos mRNA-
178 expressing cells, the area of the anteromedial domain of the OT was delineated, and the number of
179 cells was counted using Image J (National Institutes of Health).

180 **Statistics**

181 Statistical significance was tested using Prism 7 (GraphPad Software). Differences were considered
182 statistically significant at $p < 0.05$.

183

184 3 Results

185 To address whether D1 and D2 neurons in the anteromedial OT play distinct roles in attractive and
186 aversive behaviors, we used optogenetic stimulation and performed RTPP tests (Fig. 1A and B). We
187 injected AAV2-EF1a-DIO-ChETA-EYFP into the anteromedial OT of D1-Cre and D2-Cre
188 transgenic mice; AAV5-EF1a-DIO-EYFP was injected as a control for optogenetic stimulation (Fig.
189 1A). At first, we examined cell type-specificity of the Cre-mediated gene expression by the AAV
190 vector. Three weeks after injection of AAV5-EF1a-DIO-EYFP into the anteromedial OT of the D1-
191 Cre and D2-Cre mice, we performed double fluorescence immunolabeling of EYFP and D1 or D2
192 mRNAs (Fig. 1C and D). In the D1-Cre mice, 94% of the EYFP(+) neurons in the cortex-like region,
193 which were putative medium spiny neurons, expressed D1 mRNA, and 5.9% of them expressed D2
194 mRNA (n = 3 mice, Fig. 1C and D). On the other hand, 14% of the EYFP(+) neurons expressed D1
195 mRNA, and approximately 83% of them expressed D2 mRNA in the D2-Cre mice (n = 3 mice, Fig.
196 1C and E). These data confirmed that these Cre transgenic mice exhibited preferential expression of
197 Cre-dependent AAV-derived genes in the D1 and D2 neurons.

198 We then tested the hypothesis that optogenetic activation of the D1 and D2 neurons in the
199 anteromedial OT may play distinct roles in eliciting attractive and aversive behaviors using RTPP
200 tests. We activated the D1 and D2 neurons using ChETA, a type of ChR2 with faster kinetics
201 (Gunaydin et al., 2010), which possibly enabled precise timing of stimulation when mice crossed the
202 chambers. The RTPP tests revealed that D1-Cre mice expressing ChETA spent significantly longer
203 time in the photo-stimulation side (64% of the 20-min RTPP tests, n = 6 mice) than the control mice
204 (52% of the 20-min RTPP tests, n = 7 mice; unpaired *t*-test: $t = 3.261$, $df = 11$) which expressed
205 EYFP without ChETA (Fig. 2A and B). In contrast, D2-Cre mice expressing ChETA spent
206 significantly shorter time in the photo-stimulation side (28% of the 20-min RTPP tests, n = 6 mice)
207 than the control mice (51% of the 20-min RTPP tests, n = 7 mice; unpaired *t*-test: $t = 3.994$, $df = 11$)
208 (Fig. 2A and B). These data suggest that activation of the D1 and D2 neurons in the anteromedial OT
209 elicit attractive and aversive behaviors, respectively.

210 After the RTPP tests, we confirmed the neural activation of the anteromedial OT by examining
211 *c-fos* mRNA expression (Bepari et al., 2012). As expected, the ipsilateral side of the anteromedial OT
212 showed a significant increase in the number of *c-fos* expressing cells in in both ChETA-EYFP-
213 expressing D1-Cre (n = 5 mice; unpaired *t*-test: $t = 4.628$, $df = 8$) and D2-Cre (n = 6 mice; unpaired *t*-
214 test: $t = 4.427$, $df = 10$) mice (Fig. 2C and D). In contrast, no clear increase in the *c-fos* expression in
215 either control D1-Cre (n = 7 mice; unpaired *t*-test: $t = 1.239$, $df = 12$) or D2-Cre (n = 7 mice;
216 unpaired *t*-test: $t = 0.7561$, $df = 12$) mice was observed (Fig. 2C and D). These results confirmed
217 activation of the anteromedial OT by optogenetic stimulation during the RTPP tests.

218

219 4 Discussion

220 In this study, we demonstrate that cell type-specific activation of the D1 and D2 neurons in the
221 anteromedial OT elicits attractive and aversive behaviors, respectively. To achieve selective
222 manipulation of the D1 or D2 neurons, which are intermingled in the cortex-like region of the OT,
223 we used D1-Cre and D2-Cre transgenic mouse lines and Cre-dependent AAV vectors (Fig. 1A and
224 C–E). This combination enabled us to deliver AAV-derived genes preferentially to the D1 or D2
225 neurons in the anteromedial OT. Optogenetic activation of the D1 and D2 neurons in the
226 anteromedial OT induced attraction to and aversion from the photo-stimulation chamber, respectively
227 (Figs. 1B, and 2A and B). After the RTPP tests, activation of the anteromedial OT was confirmed by
228 local increase in the c-fos expression (Fig. 2C and D). These results suggest that the D1 and D2
229 neurons in the anteromedial OT are involved in eliciting attractive and aversive behaviors,
230 respectively.

231 Previous studies have shown that the anteromedial domain of the OT plays an important role
232 in the reward system. Local self-administration of cocaine into the OT and NAc revealed that the
233 anteromedial domain of the OT more robustly mediates the rewarding action of cocaine than other
234 domains of the OT and NAc (Ikemoto, 2003). Optogenetic stimulation of the dopaminergic fiber
235 from the ventral tegmental area to the medial OT elicits rewarding effects which generate place and
236 odor preference (Zhang et al., 2017a). These local manipulations should exert excitatory effect on the
237 D1 neurons via increased dopamine level in the anteromedial OT because D1 is coupled with Gs
238 (Stoof and Keibian, 1981). In line with these previous reports, our result directly demonstrates the
239 role of the D1 neurons in the anteromedial OT in eliciting attractive behavior. In contrast, D2 is
240 coupled with Gi (Stoof and Keibian, 1981). Aversive stimuli reduce tonic firings of dopaminergic
241 neurons (Ungless et al., 2004; Cohen et al., 2012) resulting in decreased ambient dopamine level at
242 the target structure, which should exert excitatory effect on the D2 neurons. Consistent with the
243 report that blunting the tonic dopamine release in the ventromedial striatum leads to conditioned
244 place aversion (Liu et al., 2008), our results reveal the role of the D2 neurons in the anteromedial OT
245 in eliciting aversive behavior. As we previously reported, neurons in the OT are activated by odor
246 cues that induce motivated behaviors. The understanding of these cell type-specific roles of the D1
247 and D2 neurons in the anteromedial OT will provide a neural basis for odor-guided adaptive
248 motivated behaviors.

249

250 **5 Conflict of Interest**

251 The authors declare that the research was conducted in the absence of any commercial or financial
252 relationships that could be construed as a potential conflict of interest.

253 **6 Author Contributions**

254 KM designed research, performed experiments, and wrote the manuscript. TK performed
255 experiments. YF, KK, AY, TH, HM, and MY contributed tools and reagents, and assisted in revision
256 of the manuscript.

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267

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355 the Inputs and Outputs of the Medial Part of the Olfactory Tubercle. *Front Neural Circuits* 11, 52.
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358 **10 Figure Legends**

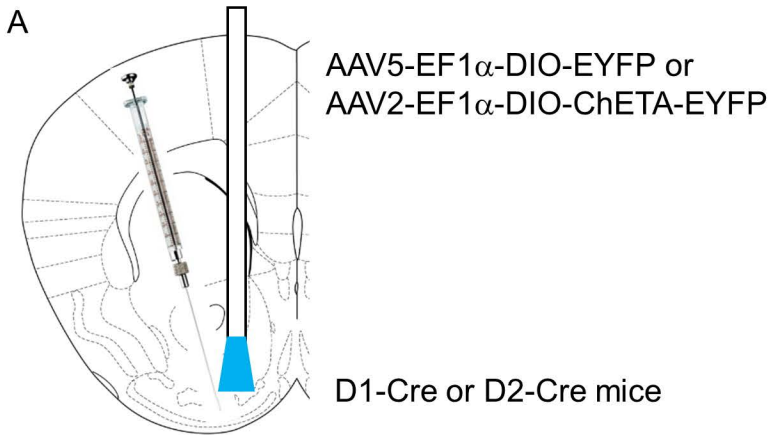
359 **Figure 1. Cell type-specific gene delivery in dopamine receptor D1- and D2-expressing neurons**
360 **in the anteromedial OT of the D1-Cre and D2-Cre mice using AAV vectors**

361 (A) Schematic diagram of cell type-specific optogenetic stimulation of the anteromedial OT. We
362 injected Cre-dependent AAVs encoding EYFP or ChETA-EYFP and implanted optic fiber canula
363 into the anteromedial OT of D1-Cre or D2-Cre mice. (B) Place preference chamber. In the RTPP
364 tests, mice were placed in either side, which was assigned as the control side (no photo-stimulation).
365 Blue light was delivered when mice were in the opposite side of the initial position. (C) and (D)
366 Confocal images of AAV-derived EYFP expressing cells (green) and D1 (upper panels) or D2 (lower
367 panels) mRNAs (red) from D1-Cre mouse (C) or D2-Cre mouse (D). Color merged panel contains
368 DAPI staining (blue). Scale bars: 20 μm . (E) Percentage of D1 or D2 mRNA expressing-cells among
369 EYFP-expressing cells in D1-Cre and D2-Cre mice. Data shows mean with SD. OT, olfactory
370 tubercle; AAV, adeno-associated virus; EYFP, enhanced yellow fluorescent protein; RTPP, real-time
371 place preference; D1, dopamine receptor D1; D2, dopamine receptor D2; DAPI, 4',6-diamidino-2-
372 phenylindole; SD, standard deviation

373 **Figure 2. Cell type-specific effect of optogenetic stimulation of the anteromedial OT in the**
374 **RTPP tests**

375 (A) Tracking data of the RTPP tests. Right side is the photo-stimulation side. (B) Percentage of time
376 spent in the photo-stimulation side in the 20-min RTPP tests. Data shows mean with SD. **, $p <$
377 0.01. (C) Images of c-fos mRNA expression in the OT coronal sections after the RTPP tests. Left
378 panels, ipsilateral; right panels, contralateral. Scale bar: 200 μm . (D) Density of c-fos mRNA-
379 expressing cells in the anteromedial OT. Data shows mean with SD. ns, not significant; *, $p <$ 0.05;
380 **, $p <$ 0.01. OT, olfactory tubercle; RTPP, real-time place preference; SD, standard deviation

Figure 1



B 20 min real-time place preference test

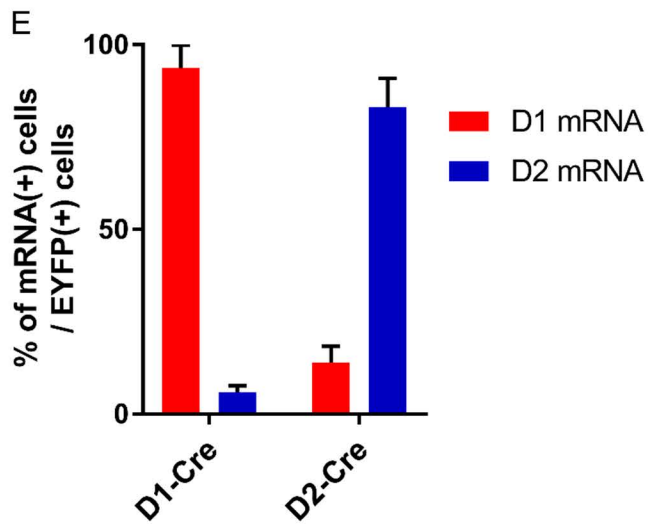
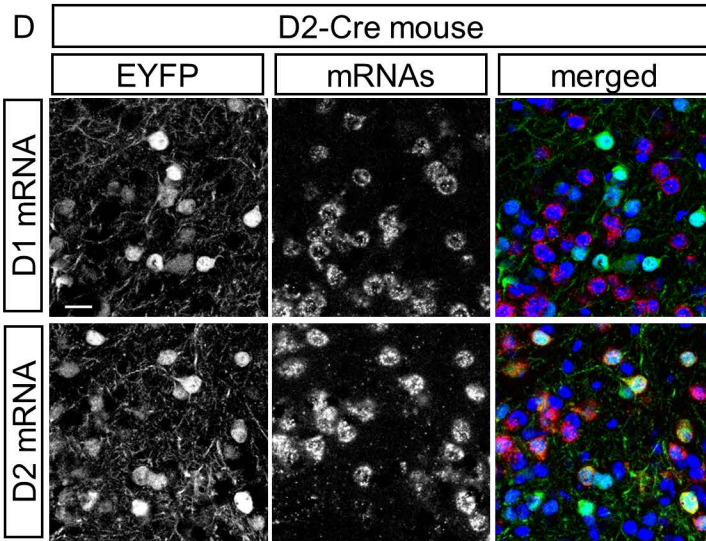
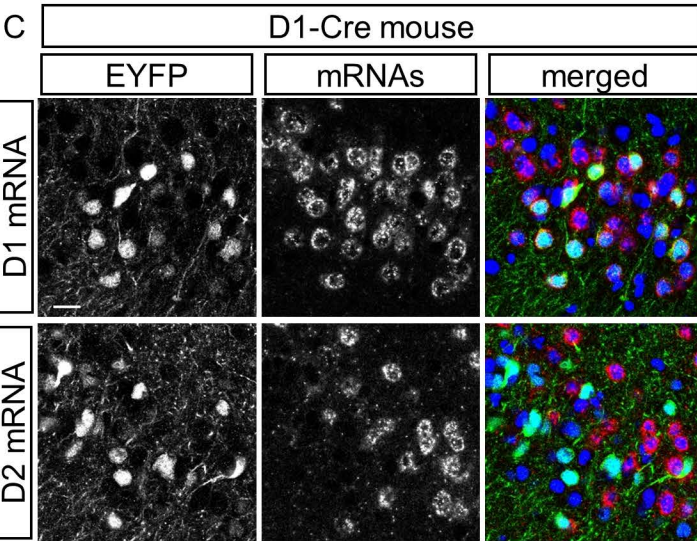
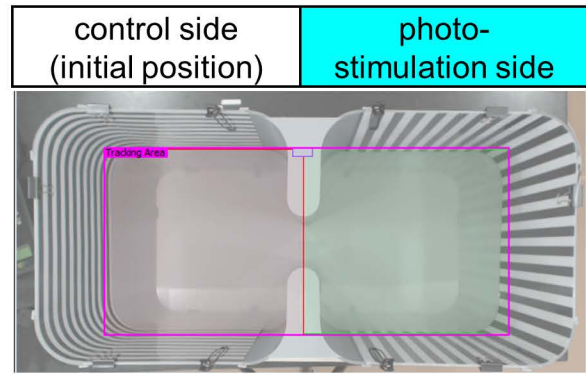


Figure 2

