1   **New and improved GRAB fluorescent sensors for monitoring dopaminergic activity *in vivo***

2

3   **Running title: Next-generation GRAB sensors for monitoring dopaminergic activity**

4

5   Fangmiao Sun[1,2,7], Jingheng Zhou[5,7], Bing Dai[6,7], Tongrui Qian[1,2], Jianzhi Zeng[1,2,3], Xuelin Li[1,2],

6   Yizhou Zhuo[1,2,3], Yajun Zhang[1,2,3], Ke Tan[1,2], Jiesi Feng[1,2,3], Hui Dong[1,2], Cheng Qian[1,2,4], Dayu

7   Lin[6]*, Guohong Cui[5]*, Yulong Li[1,2,3]*

8

9   [1]State Key Laboratory of Membrane Biology, Peking University School of Life Sciences, 100871

10   Beijing, China.

11   [2]PKU-IDG/McGovern Institute for Brain Research, 100871 Beijing, China.

12   [3]Peking-Tsinghua Center for Life Sciences, 100871 Beijing, China.

13   [4]School of Life Sciences, Tsinghua University, Beijing 100084, China.

14   [5]Neurobiology Laboratory, National Institute of Environmental Health Sciences, National Institutes

15   of Health, Research Triangle Park, NC 27709, USA.

16   [6]Neuroscience Institute, Department of Psychiatry, New York University School of Medicine, New

17   York, NY 10016, USA.

18   [7]These authors contributed equally.

19   *e-mail: Dayu.Lin@nyulangone.org; cuig@mail.nih.gov; yulongli@pku.edu.cn.

20

21   **The monoamine neuromodulator dopamine (DA) plays a critical role in the brain, and the**

22   **ability to directly measure dopaminergic activity is essential for understanding its**

23   **physiological functions. We therefore developed the first red fluorescent GPCR-activation–**

24   **based DA (GRAB$_{DA}$) sensors and optimized versions of green fluorescent GRAB$_{DA}$ sensors**

25   **following our previous studies. In response to extracellular DA, both the red and green**

26   **GRAB$_{DA}$ sensors have a large increase in fluorescence ($\Delta F/F_0$ values of 150% and 340%,**

27   **respectively), with subcellular resolution, subsecond kinetics, and nanomolar to**

28   **submicromolar affinity. Moreover, both the red and green GRAB$_{DA}$ sensors readily resolve**

29   **evoked DA release in mouse brain slices, detect compartmental DA release in live flies with**

30   **single-cell resolution, and report optogenetically elicited nigrostriatal DA release as well as**

31   **mesoaccumbens dopaminergic activity during sexual behavior in freely behaving mice.**

32   **Importantly, co-expressing red GRAB$_{DA}$ with either green GRAB$_{DA}$ or the calcium indicator**

33   **GCaMP6s provides a robust tool for simultaneously tracking neuronal activity and**

34   **dopaminergic signaling in distinct circuits *in vivo*.**

35

36   Dopamine (DA) is an essential monoamine neuromodulator produced primarily in the midbrain and

37   released throughout the central nervous system. A multitude of brain functions are regulated by DA,

38   including motor control, motivation, learning and memory, and emotional control[1-9]. Consistent with

39   these key physiological roles, altered DA signaling has been implicated in a variety of brain

40   disorders, including Parkinson's disease, addiction, schizophrenia, attention-deficit/hyperactivity

41   disorder, and posttraumatic stress disorder[10-20]. Thus, tools that can sense changes in DA

42   concentration with high spatiotemporal resolution, high specificity, and high sensitivity will greatly

43   facilitate our study of the diverse functions that the dopaminergic system plays under both

44   physiological and pathological conditions.

45   Previous techniques for measuring DA dynamics, including microdialysis, electrochemical

46   probes, reporter cells, and gene expression–based assays, lack sufficient spatiotemporal resolution

47   and/or molecular specificity[21-30]. Recently, our group[31] and Patriarchi et al.[32] independently

48   developed two series of genetically encoded, G-protein–coupled receptor (GPCR)–based DA

49   sensors called $GRAB_{DA}$ and dLight, respectively. Taking advantage of naturally occurring DA

50   receptors, these sensors convert a ligand–stabilized conformational change in the DA receptor into

51   an optical response via a conformation-sensitive fluorescent protein inserted in the receptor's third

52   intracellular loop. Our first-generation DA receptor–based sensors called $GRAB_{DA1m}$ and

53      $GRAB_{DA1h}$ were used to detect cell type–specific DA dynamics in several organisms, including

54   *Drosophila*, zebrafish, mice, and zebra finches[31,33-35]. Here, we employed semi-rational engineering

55   to modify the green fluorescent protein. The resulted second-generation sensors called $GRAB_{DA2m}$

56   and $GRAB_{DA2h}$ (abbreviated DA2m and DA2h, respectively) have a 2–3-fold higher dynamic range

57   and improved *in vivo* performance in comparison to their corresponding first-generation sensors.

58   Red fluorescent sensors have distinct and well-separated spectra from those of GFP–based sensors

59   and blue light–excitable channelrhodopsin 2 (ChR2), thus enabling the orthogonal readout of two

60   neurochemical events or simultaneous monitoring blue light–mediated activity control. Moreover,

61   red fluorescent sensors require an excitation light with a relatively longer wavelength, providing

62   additional advantages over green fluorescent proteins, including reduced phototoxicity, reduced

63   background, and deeper tissue penetration[36,37]. Starting with the dopamine D2 receptor (D_2R) and a

64   conformation-sensitive red fluorescent protein cpmApple[38], we generated a series of red fluorescent

65   DA sensors called $rGRAB_{DA1m}$ and $rGRAB_{DA1h}$ (abbreviated rDA1m and rDA1h, respectively),

66   with a dynamic range similar to the corresponding first-generation green DA sensors.

67   Here, we report the development, *in vitro* characterization, and *in vivo* application of our novel

68   red fluorescent DA sensors and second-generation green fluorescent DA sensors for monitoring DA

69   dynamics in real-time in *Drosophila* and mice in response to physiologically relevant stimuli and

70   during complex behaviors.

71

72   **Results**

73   **Development and *in vitro* characterization of DA sensors.** To develop red fluorescent DA sensors,

74   we systematically optimized the cpmApple insertion sites within the third intracellular loop of D_2R,

75   the linker sequences, and the cpmApple module itself, using both brightness and DA–induced

76   change in fluorescence ($\Delta F/F_0$) as our selection criteria. Screening a library containing over 2000

77   sensor variants revealed the sensor with the highest fluorescence response; we called this sensor

78   rDA0.5. Next, we used a rational strategy to introduce an iterative series of mutations in the D_2R

79   module of rDA0.5, generating versions with differing apparent affinities to DA. The medium-

80   affinity sensor rDA1m was generated by introducing the $K367^{6.29}L$ mutation in the receptor, and the

81   high-affinity sensor rDA1h was generated by introducing the $T205^{5.54}M$ mutation in rDA1m. Finally

82   a DA–insensitive version (rDA-mut) was generated by introducing the $C118^{3.36}A$ and $S193^{5.42}N$

83   mutations in rDA1h (Fig. 1a,b,d and Supplementary Fig. S1b,c). All three versions localized nicely

84   on the cell membrane when expressed in HEK293T cells (Fig. 1c). rDA1m and rDA1h had an EC_{50}

85   of 95 nM and 4 nM, respectively (Fig. 1d). Moreover, the application of 100 μM DA elicited a 150%

86   and 100% increase in fluorescence of rDA1m and rDA1h, respectively, which was blocked by the

87   D_2R antagonist haloperidol (Halo) (Fig. 1c,d). As expected, DA had no effect in cells expressing

88   rDA-mut, even at the highest concentration tested (Fig. 1c,d).

89   Next, we asked whether the red DA sensors undergo photoactivation when expressed in
90 HEK293T cells and cultured neurons. Previous studies showed that cpmApple–based sensors can
91 undergo photoactivation when illuminated with blue light[39-41], preventing the combined use of these
92 sensors with ChR2. We found that although cpmApple–based red fluorescent calcium indicator
93 jRGECO1a[41] had a 20% increase in fluorescence upon blue light illumination (Fig. 1e), blue light
94 had no effect on the fluorescence of either rDA1m or rDA1h, suggesting that our red fluorescent
95 DA sensors are compatible for use with blue light–mediated optogenetic activation. Moreover, we
96 found that photostability of the red DA sensors was similar to or better than several commonly used
97 red fluorescent proteins (Supplementary Fig. S1d).

98   In parallel, we optimized our first-generation green fluorescent DA sensors by performing
99 random mutagenesis at 32 sites in the cpEGFP module (Fig. 1f). Screening ~1000 variants yielded
100 DA2h, the second-generation high-affinity green fluorescent DA sensor (Fig. 1g and Supplementary
101 Fig. S1a); this sensor was then used to generate the medium-affinity DA2m and DA–insensitive
102 DA-mut versions (Fig. 1g). Compared to their corresponding first-generation DA1m and DA1h
103 sensors, the second-generation green fluorescent DA sensors had a 2–3 fold higher fluorescence
104 increase ($\Delta F/F_0$ ~340% for DA2m and ~280% for DA2h) to 100 μM DA while maintaining their
105 apparent affinity to DA, with EC_{50} values of 90 nM and 7 nM for DA2m and DA2h, respectively
106 (Fig. 1h,i). Finally, the DA–insensitive DA-mut sensor localized well on the cell membrane but did
107 not respond to DA (Fig. 1h,i).

108   We further characterized the specificity, kinetics, and downstream coupling of our newly
109 developed DA sensors. With respect to specificity, the DA–induced signals of both the red and green
110 DA sensors were blocked by the D_2R–specific antagonists Halo and eticlopride, but not the D_1R
111 antagonist SCH-23390. None of these four DA sensors responded to a variety of neurotransmitters
112 and neuromodulators (Fig. 2a). We also found that although norepinephrine (NE) is structurally
113 similar to DA, both the red and green DA sensors were 10–20 fold more selective for DA over NE
114 (Fig. 2a, inset), suggesting the high selectivity of the sensors to DA at physiologically relevant
115 concentrations[42-45].

116   We next characterized the kinetics of the DA sensors using rapid line scanning in response to a
117 local puff of DA (to measure τ_{on}) followed by Halo (to measure τ_{off}) to sensor-expressing HEK293T
118 cells. As shown in Fig. 2b and 2c, τ_{on} was <100 ms for all four DA sensors while the high-affinity
119 versions of the red and green fluorescent sensors had relatively slower off kinetics compared to their
120 corresponding medium-affinity counterparts.

121   To examine whether our DA sensors couple to downstream signaling pathways, we used the
122 luciferase complementation assay[46] and TANGO assay[47] to measure activation of the Gi (Fig. 2d)
123 and β-arrestin (Fig. 2e) pathways, respectively. When expressed in HEK293T cells, both the rDA1h
124 and DA2h sensors had virtually no downstream coupling; as a control, the wild-type D_2R showed
125 robust coupling (Fig. 2d,e). In the cultured neurons, we also found that both the red and green DA
126 sensors readily localized on the cell membrane and responded well to DA (Fig. 2f-i), supporting the
127 utility of the sensors in the nervous system.

128   Lastly, we compared the properties of the second-generation green fluorescent DA2m sensor with
129 D_1R–based dLight1.1 and dLight 1.2 sensors (Supplementary Fig. S2a-j). When expressed in
130 cultured cells, DA2m had a higher apparent affinity to DA, higher basal brightness, a higher $\Delta F/F_0$
131 response, and a higher signal-to-noise ratio (SNR) than dLight.

132   Taken together, these results indicate that our red and improved green fluorescent DA sensors are

133   highly sensitive and specific to DA, with rapid kinetics, suggesting that they will be suitable for
134   monitoring dopaminergic activity *in vivo*.

136   **Imaging of DA release in acute mouse brain slices.** Next, we examined whether our DA sensors
137   can be used to measure the release of endogenous DA in acute brain slices. We first injected AAVs
138   expressing rDA1m, rDA1h, or DA2m into the nucleus accumbens (NAc), which receives strong
139   innervation from midbrain dopaminergic neurons[48-50] (Fig. 3a,b). Two weeks after expressing the
140   sensor, we prepared acute brain slices and used 2-photon imaging combined with electrical
141   stimulation to measure stimulus–evoked DA release (Fig. 3a). Electrical stimuli delivered at 20 Hz
142   induced progressively increased fluorescence responses in the NAc, which was blocked by Halo
143   (Fig. 3c,e). We also measured the sensors' kinetics during 10 pulses applied at 100 Hz. The τ_{on} and
144   τ_{off} values are 0.08–0.15 s and 5.2–11.8 s, respectively (Fig. 3d).
145   To test whether the red fluorescent DA sensor is spectrally compatible with the green fluorescent
146   calcium sensor, we co-expressed the axon–targeted GCaMP6s[51] in the ventral tegmental area (VTA)
147   and rDA1m in the NAc, then simultaneously imaged calcium and DA in the NAc during 20 Hz
148   electrical stimulation (Fig. 3f,g). We found that the electrical stimulation evoked robust fluorescence
149   increase of both GCaMP6s and rDA1m and their magnitudes of increases were highly correlated
150   (Fig. 3k). Application of the D2R antagonist Halo blocked the rDA1m response but had no effect on
151   the GCaMP6s response (Fig. 3h-j). Taken together, these data indicate that the rDA1m, rDA1h, and
152   DA2m sensors can detect dopaminergic activity in brain slices with high specificity, sensitivity and
153   temporospatial resolution. Moreover, our results confirm that the red fluorescent DA sensors are
154   spectrally compatible with green fluorescent probes, allowing for simultaneous dual-color imaging.

156   ***In vivo* imaging of DA in *Drosophila*.** In *Drosophila*, dopaminergic activity in the mushroom body
157   (MB) has been found to be both necessary and sufficient for the associate learning between odor
158   and aversive experience, e.g. body shock[52-55]. Next, we generated a transgenic *Drosophila*
159   expressing rDA1m in Kenyon cells (KCs) in the olfactory MB and measured the fluorescence level
160   of the rDA1m sensor using *in vivo* 2-photon imaging when physiologically relevant stimuli were
161   presented (Fig. 4a,b). When we delivered either the odorant or body shock, we observed a time–
162   locked fluorescence increase in the medial lobe of the MB; this increase was blocked by pretreating
163   the animals with Halo (Fig. 4c,d) and was not observed in flies expressing the DA–insensitive rDA-
164   mut sensor. Importantly, the endogenous signal did not saturate the rDA1m sensor's response, as
165   application of 100 μM DA caused a significantly larger, sustained increase in fluorescence (Fig. 4e).
166   We then compared *in vivo* performance between the first-generation and second-generation green
167   fluorescent DA sensors in flies. Electrical stimulation of the MB medial lobe elicited robust
168   fluorescence increase in nearby DA sensor-expressing dopaminergic neurons (DANs) with a higher
169   response observed in DA2m-expressing flies (Fig. 4f,g). The temporal dynamics of the DA2m and
170   DA1m responses (τ_{on} and τ_{off}) were similar (Fig. 4l) and both responses were blocked by Halo (Fig.
171   4h-k). The spatial patterns of the responses in the MB during odorant or body shock delivery were
172   also similar between DA2m- and DA1m-expressing flies[31] while consistently higher responses were
173   observed in DA2m-expressing flies (Fig. 4m-o). In a separate experiment, we compared the *in vivo*
174   performance of DA2m with dLight1.3b, which has the highest dynamic range among the dLight
175   series of sensors, and found that DA2m produced a 3-fold larger response (Supplementary Fig. S2k-
176   o).

177   Previous studies have found that the *Drosophila* MB is innervated by the axonal neuropils from
178   15 DAN subgroups, with each lobe containing axons from one to approximately two dozens of
179   neurons[56,57,58]. We next asked whether our highly sensitive DA2m sensor could detect DA release
180   in individual MB compartments, even when the source of DA is from a single neuron. As a proof-
181   of-principle experiment, we co-expressed the red light–activated channelrhodopsin CsChrimson[59]
182   in DANs and DA2m in KCs. We then used 635 nm wavelength light to activate the only DAN
183   neuron innervating the γ2α'1 compartment or the 8–21 neurons innervating the γ5 compartment
184   while measuring DA2m fluorescence (Fig. 4p). We found that the change in DA2m fluorescence
185   was both time–locked to the CsChrimson activation and spatially confined to the respective
186   compartments, supporting functional independence among MB compartments (Fig. 4q,r).

187   To confirm that the DA2m sensor does not couple to Gi signaling *in vivo*, we used cAMP level
188   as a proxy for Gi signaling and expressed the red fluorescent cAMP sensor Pink-Flamindo[60] to
189   measure body shock–evoked increase in cAMP in KCs. We found that the presence of the DA2m
190   sensor had no effect on cAMP production (Supplementary Fig. S3). Thus, these data confirm that
191   the DA2m sensor does not affect the endogenous signaling pathways *in vivo*.

192

193   **Red and green fluorescent DA sensors can be used to detect optogenetically induced**
194   **nigrostriatal DA release in freely moving mice.** To demonstrate that our new DA sensors can be
195   used to measure dopaminergic activity in freely moving animals, we measured DA dynamics in the
196   mouse dorsal striatum, which receives motor control–related nigrostriatal dopaminergic projections
197   from the substantia nigra pars compacta (SNc)[61], by expressing the optogenetic tool C1V1[62] in the
198   SNc and various DA sensors in the dorsal striatum. To provide a frame of reference for the red and
199   green fluorescent sensors, we also co-expressed EGFP or tdTomato, respectively, in the dorsal
200   striatum (Fig. 5a,b). C1V1–mediated optogenetic activation of DANs in the SNc elicited a robust
201   transient increase in DA sensor fluorescence in the dorsal striatum (Fig. 5e,g,i,k). Importantly, the
202   signal was prolonged by the DA transporter blocker methylphenidate and blocked by the D_2R
203   antagonist eticlopride (Fig. 5e-l); in contrast, the signal was unaffected by the NE transporter blocker
204   desipramine and the α_2-adrenergic receptor antagonist yohimbine (Supplementary Fig. S4). As a
205   control, the DA–insensitive sensor rDA-mut did not respond to C1V1–mediated optogenetic
206   activation (Fig. 5c,d). Finally, both EGFP fluorescence and tdTomato fluorescence were unchanged
207   throughout the experiments, indicating a lack of movement–related artifacts during recording. Thus,
208   our new DA sensors can detect optogenetically induced DA release in freely moving mice with high
209   sensitivity and high molecular specificity.

210

211   **Detection of dopaminergic activity in the mouse NAc during sexual behavior.** We previously
212   showed[31] that dopaminergic activity can be measured during male sexual behavior[4]. To ask whether
213   our improved DA2h performs better than the DA1h sensor in reporting real-time DA dynamics in
214   freely moving mice, we expressed DA1h in one side of the NAc and DA2h in the contralateral NAc,
215   and performed bilateral fiber photometry recording during mating (Fig. 6a,b). We found that the
216   DA1h and DA2h signals were closely correlated in time. Consistent with the improved performance
217   of DA2h in other systems, the DA2h sensor had a significantly higher fluorescence change ($\Delta F/F_0$)
218   than DA1h during various stages of sexual behavior (Fig. 6c-f).

219   We next compared the performance of red fluorescent rDA1m sensor with that of the best green
220   fluorescent DA sensor, DA2h. We co-injected viruses expressing rDA1m and DA2h into the NAc

221   core and performed dual-color fiber photometry recording three weeks after virus injection (Fig.
222   6g,h). We found that despite the dynamic range ($\Delta F/F_0$) of the rDA1m is smaller than that of DA2h
223   (Supplementary Fig. S5 and S6), rDA1m and DA2h detected qualitatively similar DA release during
224   sexual behavior when the responses are Z scores (Fig. 6i,k,l). The moment-to-moment correlation
225   coefficient between rDA1m and DA2h is similar to that between DA1h and DA2h (Fig. 6d,j).
226   Importantly, we did not observe crosstalk between the red and green DA sensors as no signal in the
227   red channel was detected when only 470 nm light was delivered and *vice versa* (Supplementary Fig.
228   S6). Taken together, the rDA1m is capable of detecting DA release *in vivo* during natural behaviors
229   and the behavior–related DA responses detected by the red and green DA sensors are qualitatively
230   similar.
231
232   **Discussion**
233   Here, we report the development and characterization of a new set of genetically encoded DA
234   sensors. Moreover, we show that these sensors can be used to measure DA release in mouse brain
235   slices, as well as in the *Drosophila* olfactory system and the NAc in freely moving mice during
236   sexual behavior. Importantly, these sensors can report DA release evoked by electrical stimulation,
237   optogenetic activation, and various physiologically relevant stimuli and behaviors. The availability
238   of both high-affinity and medium-affinity versions provides the opportunity to probe DA dynamics
239   over a broad range of concentrations. Moreover, the DA2m sensor provides an ideal balance of
240   sensitivity, dynamic range, and brightness, making it a powerful tool for monitoring DA dynamics
241   *in vivo* with a high signal-to-noise ratio in several models, including flies and mice[63,64].
242   DA has long been regarded as a neuromodulator that can diffuse broadly and exert its effects
243   over a large volume with both slow and fast kinetics as well as tonic and phasic release patterns.
244   However, several key questions regarding dopaminergic activity remain unanswered. For example,
245   how are the release and postsynaptic effects of DA organized at the spatiotemporal level? How does
246   DA release at a given time affect synaptic plasticity and the transfer of information to specific
247   neuronal subpopulations? How is dopaminergic activity regulated by various neuromodulators?
248   Addressing these fundamental questions requires cell type–specific tools that can be used to monitor
249   DA dynamics in real time with high spatial resolution, high molecular specificity, high sensitivity,
250   and rapid kinetics, thereby improving our understanding of the role that DA plays in both health and
251   disease. In this respect, our DA sensors provide a suitable tool that can be combined with other
252   techniques such as optogenetics, calcium imaging, and electrophysiology.
253   Neuromodulatory neurons such as dopaminergic neurons send their projections throughout the
254   brain, and each brain region can receive several neuromodulators. These various neurochemical
255   pathways closely interact in order to regulate behavior in a state–dependent and highly cooperative
256   manner. Thus, our red fluorescent rGRAB$_{DA}$ sensors provide a new strategy for studying the
257   interplay between various neuromodulators and neurotransmitters, given that our red fluorescent
258   DA sensors are spectrally compatible with green fluorescent sensors such as the GRAB$_{ACh}$
259   sensor[65,66] and GRAB$_{NE}$ sensor[67], allowing the simultaneous monitoring of DA with acetylcholine
260   and norepinephrine, respectively. In addition, combining the red fluorescent DA sensors with green
261   fluorescent GCaMPs can provide new insights regarding the putative role that DA plays in
262   regulating pre- and/or post-neuronal activities, as well as the function of target neural circuits and
263   behaviors. Finally, several studies have shown that the GRAB–based sensor strategy can be easily
264   used to create genetically encoded sensors based on a wide range of G-protein–coupled

265 receptors[31,65-67], leading to a robust and versatile multi-color toolbox that can be used to create

266 comprehensive functional maps of neurochemical activity.

267

268 **Methods**

269 **Animals.** Postnatal 0-day-old (P0) Sprague-Dawley rats (Beijing Vital River Laboratory) and adult

270 (P42–90) wild-type C57BL/6N (Beijing Vital River Laboratory), wild-type C57BL/6J (Charles

271 River Laboratories), and DAT-IRES-Cre mice (Jackson Laboratory, stock number 06660) were used

272 in this study. All animals were housed in a temperature-controlled room with a 12 h/12 h light-dark

273 cycle. All procedures for animal surgery, maintenance, and behavior were performed using protocols

274 that were approved by the respective animal care and use committees at Peking University, New

275 York University, and the US National Institutes of Health.

276 The transgenic *Drosophila* lines UAS-rDA1m, UAS-rDA-mut, UAS-DA2m, UAS-dLight1.3b,

277 and UAS-Pink-Flamindo were generated using Phi-C31–directed integration into attp40 or

278 VK00005 at the Core Facility of Drosophila Resource and Technology, Shanghai Institute of

279 Biochemistry and Cell Biology, Chinese Academy of Sciences. The following *Drosophila* lines were

280 also used in this study: UAS-DA1m (BDSC: 80047), R13F02-Gal4 (BDSC: 49032), 30y-Gal4, and

281 TH-Gal4-3p3-RFP[68] (all gifts from Yi Rao, Peking University, Beijing); MB312C-Gal4 (Fly light:

282 2135360); MB315C-Gal4 (Fly light: 2135363); and UAS-CsChrimson-mCherry (a gift from Chuan

283 Zhou, Institute of Zoology, Chinese Academy of Sciences, Beijing). The flies were raised on

284 standard cornmeal-yeast medium at 25°C, 70% relative humidity, and a 12 h/12 h light-dark cycle.

285 Adult female flies within 2 weeks after eclosion were used for fluorescence imaging.

286

287 **Molecular Biology.** DNA fragments were generated using PCR amplification with primers

288 (TSINGKE Biological Technology) containing 30 bp of overlap. The fragments were then

289 assembled into plasmids using Gibson assembly[69]. All plasmid sequences were verified using

290 Sanger sequencing (TSINGKE Biological Technology). For characterization in HEK293T cells, the

291 genes expressing the red and green DA sensors were cloned into the pDisplay vector, with an IgK

292 leader sequence inserted upstream of the sensor gene. The IRES-EGFP-CAAX gene (for red DA

293 sensors) or IRES-mCherry-CAAX gene (for green DA sensors) was attached downstream of the

294 sensor gene and was used as a membrane marker and to calibrate the fluorescence signal intensity.

295 Site–directed mutagenesis was performed using primers containing randomized NNB codons (48

296 codons in total, encoding 20 possible amino acids) or defined codons on the target sites. For

297 characterization in cultured neurons, the sensor genes were cloned into the pAAV vector under the

298 control of the human synapsin promoter (hSyn). To generate stable cell lines expressing wild-type

299 D_2R, rDA1h, or DA2h, we generated a vector called pPacific, containing various elements,

300 including 30 TR, the myc tag gene, a 2A sequence, the mCherry gene, the puromycin gene, and 50

301 TR; the genes were then cloned into the pPacific vector using Gibson assembly. Two mutations

302 (S103P and S509G) were introduced in pCS7-PiggyBAC (ViewSolid Biotech) to generate a

303 hyperactive piggyBac transposase[70] for generating stable cell lines. For the TANGO assay, the wild-

304 type D_2R, rDA1h, or DA2h genes were cloned into the pTango vector[47]. For the luciferase

305 complementation assay, we replaced the β_2AR gene in the β_2AR-Smbit construct[46] with the wild-

306 type D_2R, rDA1h, or DA2h genes. To generate transgenic fly lines, the respective sensor genes were

307 cloned into the pUAST vector, which was then used for P-element–mediated random insertion.

308

309 **Preparation and fluorescence imaging of cultured cells.** HEK293T cells were cultured in DMEM

310 (Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco) and 1% penicillin-streptomycin

311 (Gibco) at 37°C in 5% CO_2. The cells were plated on 96-well plates or 12 mm glass coverslips in

312   24-well plates and grown to 60% confluence for transfection. For transfection, the cells were
313   incubated in a mixture containing 1 μg DNA and 3 μg PEI for 6 h. Fluorescence imaging was
314   performed 24–48 h after transfection. Rat cortical neurons were prepared from P0 Sprague-Dawley
315   rat pups (Beijing Vital River Laboratory). In brief, cortical neurons were dissociated from dissected
316   rat brains in 0.25% Trypsin-EDTA (Gibco), plated on 12 mm glass coverslips coated with poly-D-
317   lysine (Sigma-Aldrich), and cultured in Neurobasal medium (Gibco) containing 2% B-27
318   supplement (Gibco), 1% GlutaMAX (Gibco), and 1% penicillin-streptomycin (Gibco) at 37℃ in 5%
319   CO_2. The neurons were transfected with an AAV expressing rDA1m, rDA1h, DA2m, DA2h, or
320   dLight1.1 (Vigene Biosciences) after 7–9 days in culture, and fluorescence imaging was performed
321   3–7 days after transfection.

322   Cultured cells were imaged using an inverted Ti-E A1 confocal microscope (Nikon) and the Opera
323   Phenix high-content screening system (PerkinElmer). The confocal microscope was equipped with
324   a 40×/1.35 NA oil-immersion objective, a 488 nm laser, and a 561 nm laser. During fluorescence
325   imaging, the cells were either bathed or perfused in a chamber containing Tyrode's solution
326   consisting of (in mM): 150 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose (pH 7.4).
327   Solutions containing various concentrations of drugs of DA (Sigma-Aldrich) and/or 1 μM Halo
328   (Tocris), SCH-23390 (Tocris), Etic (Tocris), L-DOPA (Abcam), 5-HT (Tocris), histamine (Tocris),
329   Glu (Sigma-Aldrich), GABA (Tocris), Ado (Tocris), ACh (Solarbio), NE (Tocris), Tyr (Sigma-
330   Aldrich), or Oct (Tocris) were delivered via a custom–made perfusion system or via bath application.
331   Between experiments, the chamber was thoroughly cleaned with 75% ethanol, 3% hydrogen
332   peroxide, and Tyrode's solution. GFP fluorescence was collected using a 525/50 nm emission filter,
333   and RFP fluorescence was collected using a 595/50 nm emission filter. Photostability was measured
334   under 1-photon illumination (confocal microscopy) using a 488 nm laser at 350 μW, and
335   photostability was measured under 2-photon illumination using a 920 nm laser at 27.5 mW.
336   Photobleaching was applied to the entire sensor-expressing HEK293T cell at an area of 200 μm^2.
337   Blue light–mediated photoactivation was measured using a 488 nm laser at 350 μW. The Opera
338   Phenix high-content screening system was equipped with a 60×/1.15 NA water-immersion objective,
339   a 488 nm laser, and a 561 nm laser. GFP fluorescence was collected using a 525/50 nm emission
340   filter, and RFP fluorescence was collected using a 600/30 nm emission filter. Where indicated, the
341   culture medium was replaced with 100 μl Tyrode's solution containing various concentrations of
342   the indicated drugs. The red and green sensors' fluorescence intensity was calibrated using EGFP
343   and mCherry, respectively.

344

345   **Spectra measurements.** HEK293T cells stably expressing rDA1m, rDA1h, or DA2h were
346   harvested and transferred to a 96-well plate in the absence or presence of 100 μM DA, and excitation
347   and emission spectra were measured at 5 nm increments using a Safire2 multi-mode plate reader
348   (Tecan).

349

350   **Luciferase complementation assay.** The luciferase complementation assay was performed as
351   previously described[46]. In brief, 24–48 h after transfection, HEK293T cells expressing rDA1h or
352   DA2h were washed in PBS, harvested by trituration, and transferred to 96-well plates. DA at various
353   concentrations (ranging from 1 nM to 100 μM) was applied to the cells, and furimazine (NanoLuc
354   Luciferase Assay, Promega) was then applied to a final concentration of 5 μM, after which
355   luminescence was measured using a Victor X5 multi-label plate reader (PerkinElmer).

356

357 **TANGO assay.** DA was applied at various concentrations (ranging from 0.1 nM to 10 μM) to a
358 reporter cell line stably expressing a tTA-dependent luciferase reporter and a β-arrestin2-TEV fusion
359 gene[47] transfected to express wild-type D_2R, rDA1h, or DA2h. The cells were then cultured for 12
360 h to allow for luciferase expression. Bright-Glo (Fluc Luciferase Assay System, Promega) was then
361 applied to a final concentration of 5 μM, and luminescence was measured using a VICTOR X5
362 multi-label plate reader (PerkinElmer).

363

364 **Preparation and fluorescence imaging of acute brain slices.** Wild-type adult (P42-56) C57BL/6N
365 mice were anesthetized with an intraperitoneal injection of 2,2,2-tribromoethanol (Avertin, 500
366 mg/kg body weight, Sigma-Aldrich), and then placed in a stereotaxic frame for AAV injection using
367 a microsyringe pump (Nanoliter 2000 Injector, WPI). In Fig. 5a-e, AAVs expressing hSyn-rDA1m,
368 hSyn-rDA1h, or hSyn-DA2m (Vigene Biosciences) were injected (400 nl per injection site) into the
369 NAc using the following coordinates: AP: +1.4 mm relative to Bregma, ML: ±1.2 mm relative to
370 Bregma, depth: 4.0 mm from the dura. In Fig. 5f-k, the AAV expressing hSyn-rDA1m (Vigene
371 Biosciences) was injected (400 nl per injection site) into the NAc using the coordinates listed above,
372 and the AAV expressing hSyn-axon-GCaMP6s (BrainVTA) was injected (400 nl per injection site)
373 into the VTA using the following coordinates: AP: -3.2 mm relative to Bregma, ML: ±0.5 mm
374 relative to Bregma, depth: 4.1 mm from the dura.
375   Two weeks after virus injection, the mice were anesthetized with an intraperitoneal injection of
376 Avertin (500 mg/kg body weight) and perfused with ice-cold oxygenated slicing buffer containing
377 (in mM): 110 choline-Cl, 2.5 KCl, 1 NaH_2PO_4, 25 $NaHCO_3$, 7 $MgCl_2$, 25 glucose, and 0.5 $CaCl_2$.
378 The brains were immediately removed and placed in ice-cold oxygenated slicing buffer. The brains
379 were sectioned into 300 μm thick slices using a VT1200 vibratome (Leica), and the slices were
380 incubated at 34°C for at least 40 min in oxygenated artificial cerebrospinal fluid (ACSF) containing
381 (in mM): 125 NaCl, 2.5 KCl, 1 NaH_2PO_4, 25 $NaHCO_3$, 1.3 $MgCl_2$, 25 glucose, and 2 $CaCl_2$. For
382 fluorescence imaging, the slices were transferred to an imaging chamber and placed under an
383 FV1000MPE 2-photon microscope (Olympus) equipped with a 25×/1.05 NA water-immersion
384 objective and a mode–locked Mai Tai Ti: Sapphire laser (Spectra-Physics). A 950 nm laser was used
385 to excite rDA1m and rDA1h, and fluorescence was collected using a 575–630 nm filter. A 920 nm
386 laser was used to excite DA2m, and fluorescence was collected using a 495–540 nm filter. For
387 electrical stimulation, a bipolar electrode (cat. number WE30031.0A3, MicroProbes for Life
388 Science) was positioned near the core of the NAc using fluorescence guidance. Fluorescence
389 imaging and electrical stimulation were synchronized using an Arduino board with custom-written
390 programs. All images collected during electrical stimulation were recorded at a frame rate of 0.1482
391 s/frame with 128×96 pixels per frame. The stimulation voltage was 4–6 V, and the duration of each
392 stimulus was 1 ms. Drugs were applied to the imaging chamber by perfusion at a flow rate at 4
393 ml/min.

394

395 **Fluorescence imaging of transgenic flies.** Adult female flies (within 2 weeks after eclosion) were
396 used for fluorescence imaging. For imaging, the fly was mounted to a customized chamber using
397 tape such that the antennae and abdomen were exposed to the air. A section of rectangular cuticle
398 between the eyes, as well as the air sacs and fat bodies, were then removed to expose the brain,
399 which was bathed in adult hemolymph-like solution (AHLS) containing (in mM): 108 NaCl, 5 KCl,

5 HEPES, 5 trehalose, 5 sucrose, 26 NaHCO$_3$, 1 NaH$_2$PO$_4$, 2 CaCl$_2$, and 1–2 MgCl$_2$. Fluorescence imaging was performed using an FV1000MPE 2-photon microscope (Olympus) equipped with a 25×/1.05 NA water-immersion objective and a mode–locked Mai Tai Ti: Sapphire laser (Spectra-Physics). A 950 nm laser was used to excite rDA1m and Pink-Flamindo, and a 575–630 nm filter was used to collect the fluorescence signals. A 930 nm laser was used to excite DA2m and dLight1.3b, and a 495–540 nm filter was used to collect the fluorescence signals. For odorant stimulation, isoamyl acetate (Sigma-Aldrich) was first diluted 200-fold in mineral oil in a bottle and then diluted 5-fold in air, which was then delivered to the fly's antennae at a rate of 1000 ml/min. Halo (Tocris) was added directly to the AHLS at the final concentration. For electrical stimulation, a glass electrode (0.2 MΩ resistance) was placed in the olfactory mushroom body in the vicinity of dopaminergic neurons, and the stimulation voltage was set at 20–80 V. For body shock, two wires were attached to the fly's abdomen, and 60 V electrical pulses (500 ms duration) were delivered. For DA perfusion, a patch of the blood-brain barrier was carefully removed using tweezers, and AHLS containing 100 μM DA was applied to exchange the normal bath solution. For optogenetic stimulation, a 200 mW 635 nm laser (Changchun Liangli photoelectricity Ltd.) was used to deliver light to the fly brain via an optical fiber. An Arduino board with custom–written programs was used to synchronize the stimulation and fluorescence imaging. The sampling rate during odorant stimulation, electrical stimulation, body shock, and DA perfusion was 6.7 Hz, 12 Hz, 6.7 Hz, and 1 Hz, respectively.

Fiber photometry recording of optogenetically induced DA release in freely moving mice. Adult (P42–56) DAT-IRES-Cre mice (Jackson Laboratory, stock number 06660) were anesthetized with isoflurane and placed in a stereotaxic frame for AAV injection. AAVs expressing hSyn-rDA1m, hSyn-rDA1h, hSyn-rDA-mut, hSyn-DA2m, or hSyn-DA2h (Vigene Biosciences) as well as hSyn-EGFP (Addgene, cat. number 50465) or hSyn-tdTomato (a gift from Dr. Yakel's Lab) were injected (1 μl per site) into the dorsal striatum using the following coordinates: AP: -0.5 mm relative to Bregma, ML: ±2.4 mm relative to Bregma, depth: 2.2 mm from the dura. The AAV expressing Ef1α-DIO-C1V1-YFP (NIEHS Viral Vector Core) was injected (500 nl per site) into the SNc using the following coordinates: AP: -3.1 mm relative to Bregma, ML: ±1.5 mm relative to Bregma, depth: 4.0 mm from the dura. Optical fibers (105 μm core/125 μm cladding) were implanted in the dorsal striatum and SNc 4 weeks after AAV injection. Fiber photometry recording in the dorsal striatum was performed using a 488 nm laser at 50 μW for DA2m and DA2h, a 488 nm laser at 1 μW and a 561 nm laser at 50 μW for rDA1m, rDA1h, and rDA-mut. C1V1 in the SNc was stimulated using a 561 nm laser at 9.9 mW. The measured emission spectra were fitted using a linear unmixing algorithm (https://www.niehs.nih.gov/research/atniehs/labs/ln/pi/iv/tools/index.cfm). To evoke C1V1–mediated DA release, pulse trains (10 ms pulses at 20 Hz for 1 s) were delivered to the SNc using a 561 nm laser at 9.9 mW. To avoid signal decay, the excitation lasers were controlled using an optical shutter (Thorlabs) in which the shutter was turned on 10 s before the 561 nm pulse trains and turned off 35 s after stimulation.

Fiber photometry recording of DA dynamics in the NAc during sexual behavior. Adult (P60–90) wild-type male C57BL/6J mice (Charles River Laboratories) were anesthetized with isoflurane and placed in a stereotaxic frame for AAV injection. AAVs expressing hSyn-rDA1m or hSyn-DA2h (Vigene Biosciences) were injected (80–140 nl per site) into the NAc using the following

444   coordinates: AP: +0.98 mm relative to Bregma, ML: ±1.2 mm relative to Bregma, depth: 4.6 mm
445   from the dura. For the co-expression of rDA1m and DA2h in Fig. 6g-l, AAVs expressing hSyn-
446   rDA1m and hSyn-DA2h were injected at a 1:1 ratio. After AAV injection, optical fibers (400 μm
447   diameter) were implanted in the NAc, and fiber photometry recording was performed two weeks
448   after AAV injection. The setups for bilateral recording and dual-color recording are shown in Fig.
449   6a and Fig. 6g, respectively. In brief, a 311 Hz 472/30 nm filtered LED (Thorlabs) at 30 μW was
450   used to excite DA1h and DA2h, and a 400 Hz 590/20 nm filtered LED (Thorlabs) at 30 μW was
451   used to excite rDA1m. A 535/50 nm filter was used to collect the fluorescence signal from DA1h
452   and DA2h, and a 524/628-25 nm dual-band bandpass filter was used to collected the fluorescence
453   signal from rDA1m and DA2h during the dual-color recording. The various sexual behaviors are
454   defined as previously described[31] following published conventions[71]. For immunofluorescence, the
455   mice were anesthetized and then perfused with 4% paraformaldehyde (PFA). The brains were
456   removed, fixed in 4% PFA for 4 h, and then cryoprotected in 20% (w/v) sucrose for 24 h. The brains
457   were then embedded in tissue-freezing medium and sectioned into 60 μm thick slices using a
458   CM1900 cryostat (Leica). rDA1m was immunostained using a rabbit anti-RFP antibody (1:1000,
459   Takara, cat. number 632496) followed by a Cy3-conjugated donkey anti-rabbit secondary antibody
460   (1:1000, Jackson ImmunoResearch, cat. number 113713). DA1h and DA2h were immunostained
461   using a chicken anti-GFP antibody (1:1000, Abcam, cat. number ab13970) followed by an Alexa
462   488-conjugated donkey anti-chicken secondary antibody (1:1000, Jackson ImmunoResearch, cat.
463   number 116967).

464

465   **Quantification and statistical analysis.** Imaging data from cultured cells, acute brain slices, and
466   transgenic flies were processed using ImageJ software (NIH). Pseudocolor images were generated
467   using custom–written programs. The fluorescence response ($\Delta F/F_0$) was calculated using the
468   formula $[(F–F_0)/F_0]$, in which F_0 is the baseline fluorescence signal. The signal-to-noise ratio (SNR)
469   was calculated as the peak response divided by the standard error of the baseline fluorescence
470   fluctuation. Summary data are presented as the mean ± s.e.m., and group data were analyzed using
471   the Student's t-test, one-way ANOVA (Supplementary Fig. S5) or two-way ANOVA (Fig. 6). Where
472   indicated, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and n.s., not significant ($p > 0.05$).

473

474   **Data and software availability.** Plasmids expressing the sensors used in this study were deposited
475   at Addgene (https://www.addgene.org/Yulong_Li/). The custom–written programs will be provided
476   upon request to the lead contact, Yulong Li (Yulongli@pku.edu.cn).

477

478   **Acknowledgments**

for sharing the 2-photon microscope and Xiaoguang Lei at PKU-CLS for providing support for the Opera Phenix high-content screening system.

Author contributions

Y.L. supervised the study. F.S., Y.L. designed the study. F.S., Y. Zhuo, and Y. Zhang performed the experiments related to developing, optimizing, and characterizing the sensors in cultured HEK293T cells and neurons with help from J.F., H.D. and C.Q.. F.S. and T.Q. performed the surgery and 2-photon imaging experiments related to the validation of the sensors in acute brain slices. X.L., K.T., and J. Zeng performed the 2-photon imaging experiments in transgenic flies. J. Zhou performed the fiber photometry recordings during optogenetics in freely moving mice under the supervision of G.C.. B.D. performed the fiber photometry recordings in the mouse NAc during sexual behavior under the supervision of D.L.. All authors contributed to the data interpretation and analysis. F.S. and Y.L. wrote the manuscript with input from all authors.

Competing interests

F.S. and Y. L. have filed patent applications whose value might be affected by this publication.

References

1   Björklund, A. & Dunnett, S. B. Dopamine neuron systems in the brain: an update. *Trends in neurosciences* **30**, 194-202 (2007).

2   Wise, R. A. Dopamine and reward: the anhedonia hypothesis 30 years on. *Neurotoxicity research* **14**, 169-183 (2008).

3   Ikemoto, S. Brain reward circuitry beyond the mesolimbic dopamine system: a neurobiological theory. *Neuroscience & biobehavioral reviews* **35**, 129-150 (2010).

4   Berridge, K. C. & Robinson, T. E. What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? *Brain research reviews* **28**, 309-369 (1998).

5   Schultz, W. Dopamine reward prediction-error signalling: a two-component response. *Nature Reviews Neuroscience* **17**, 183 (2016).

6   Wise, R. A. Dopamine, learning and motivation. *Nature reviews neuroscience* **5**, 483-494 (2004).

7   Holroyd, C. B. & Coles, M. G. The neural basis of human error processing: reinforcement learning, dopamine, and the error-related negativity. *Psychological review* **109**, 679 (2002).

8   Nieoullon, A. Dopamine and the regulation of cognition and attention. *Progress in neurobiology* **67**, 53-83 (2002).

9   Graybiel, A. M., Aosaki, T., Flaherty, A. W. & Kimura, M. The basal ganglia and adaptive motor control. *Science* **265**, 1826-1831 (1994).

10   Lotharius, J. & Brundin, P. Pathogenesis of Parkinson's disease: dopamine, vesicles and α-synuclein. *Nature Reviews Neuroscience* **3**, 932-942 (2002).

11   Di Chiara, G. & Imperato, A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proceedings of the National Academy of Sciences* **85**, 5274-5278 (1988).

12   Cook Jr, E. H. *et al.* Association of attention-deficit disorder and the dopamine transporter gene. *American journal of human genetics* **56**, 993 (1995).

13   Howes, O. D. & Kapur, S. The dopamine hypothesis of schizophrenia: version III—the final common pathway. *Schizophrenia bulletin* **35**, 549-562 (2009).

532 14 Arnsten, A. F. & Dudley, A. G. Methylphenidate improves prefrontal cortical cognitive function through
533   α2 adrenoceptor and dopamine D1 receptor actions: Relevance to therapeutic effects in Attention Deficit
534   Hyperactivity Disorder. *Behavioral and Brain Functions* **1**, 2 (2005).

535 15 Deutch, A. Y. & Young, C. D. A model of the stress-induced activation of prefrontal cortical dopamine
536   systems: Coping and the development of post-traumatic stress disorder. (1995).

537 16 Grace, A. A. Dysregulation of the dopamine system in the pathophysiology of schizophrenia and
538   depression. *Nature Reviews Neuroscience* **17**, 524 (2016).

539 17 Granon, S. *et al.* Enhanced and impaired attentional performance after infusion of D1 dopaminergic
540   receptor agents into rat prefrontal cortex. *Journal of neuroscience* **20**, 1208-1215 (2000).

541 18 Lee, J. C., Wang, L. P. & Tsien, J. Z. Dopamine rebound-excitation theory: putting brakes on PTSD.
542   *Frontiers in psychiatry* **7**, 163 (2016).

543 19 Okubo, Y. *et al.* Decreased prefrontal dopamine D1 receptors in schizophrenia revealed by PET. *Nature*
544   **385**, 634-636 (1997).

545 20 Pitman, R. K. *et al.* Biological studies of post-traumatic stress disorder. *nature Reviews neuroscience* **13**,
546   769-787 (2012).

547 21 Chefer, V. I., Thompson, A. C., Zapata, A. & Shippenberg, T. S. Overview of brain microdialysis. *Current*
548   *protocols in neuroscience* **47**, 7.1. 1-7.1. 28 (2009).

549 22 Tidey, J. W. & Miczek, K. A. Social defeat stress selectively alters mesocorticolimbic dopamine release:
550   an in vivo microdialysis study. *Brain research* **721**, 140-149 (1996).

551 23 Robinson, D. L., Venton, B. J., Heien, M. L. & Wightman, R. M. Detecting subsecond dopamine release
552   with fast-scan cyclic voltammetry in vivo. *Clinical chemistry* **49**, 1763-1773 (2003).

553 24 Robinson, D. L., Hermans, A., Seipel, A. T. & Wightman, R. M. Monitoring rapid chemical
554   communication in the brain. *Chemical reviews* **108**, 2554-2584 (2008).

555 25 Jaquins-Gerstl, A. & Michael, A. C. A review of the effects of FSCV and microdialysis measurements on
556   dopamine release in the surrounding tissue. *Analyst* **140**, 3696-3708 (2015).

557 26 Muller, A., Joseph, V., Slesinger, P. A. & Kleinfeld, D. Cell-based reporters reveal in vivo dynamics of
558   dopamine and norepinephrine release in murine cortex. *Nature methods* **11**, 1245 (2014).

559 27 Inagaki, H. K. *et al.* Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals
560   appetite control of sugar sensing. *Cell* **148**, 583-595 (2012).

561 28 Lee, D. *et al.* Temporally precise labeling and control of neuromodulatory circuits in the mammalian brain.
562   *Nature methods* **14**, 495 (2017).

563 29 Kim, M. W. *et al.* Time-gated detection of protein-protein interactions with transcriptional readout. *Elife*
564   **6**, e30233 (2017).

565 30 Barnea, G. *et al.* The genetic design of signaling cascades to record receptor activation. *Proceedings of the*
566   *National Academy of Sciences* **105**, 64-69 (2008).

567 31 Sun, F. *et al.* A Genetically Encoded Fluorescent Sensor Enables Rapid and Specific Detection of
568   Dopamine in Flies, Fish, and Mice. *Cell* **174**, 481-496 e419, doi:10.1016/j.cell.2018.06.042 (2018).

569 32 Patriarchi, T. *et al.* Ultrafast neuronal imaging of dopamine dynamics with designed genetically encoded
570   sensors. *Science* **360**, 1420-+, doi:ARTN eaat4422
571 10.1126/science.aat4422 (2018).

572 33 Tanaka, M., Sun, F., Li, Y. & Mooney, R. A mesocortical dopamine circuit enables the cultural transmission
573   of vocal behaviour. *Nature* **563**, 117-120, doi:10.1038/s41586-018-0636-7 (2018).

574 34 Zhou, M. *et al.* Suppression of GABAergic neurons through D2-like receptor secures efficient conditioning
575   in Drosophila aversive olfactory learning. *Proc Natl Acad Sci U S A* **116**, 5118-5125,

576   doi:10.1073/pnas.1812342116 (2019).

577 35 Handler, A. *et al.* Distinct Dopamine Receptor Pathways Underlie the Temporal Sensitivity of Associative
578   Learning. *Cell* **178**, 60-75 e19, doi:10.1016/j.cell.2019.05.040 (2019).

579 36 Shu, X. *et al.* Mammalian expression of infrared fluorescent proteins engineered from a bacterial
580   phytochrome. *Science* **324**, 804-807 (2009).

581 37 Alford, S. C., Wu, J., Zhao, Y., Campbell, R. E. & Knöpfel, T. Optogenetic reporters. *Biology of the Cell*
582   **105**, 14-29 (2013).

583 38 Zhao, Y. *et al.* An expanded palette of genetically encoded Ca(2)(+) indicators. *Science* **333**, 1888-1891,
584   doi:10.1126/science.1208592 (2011).

585 39 Wu, J. *et al.* Improved orange and red Ca(2)+/- indicators and photophysical considerations for optogenetic
586   applications. *ACS Chem Neurosci* **4**, 963-972, doi:10.1021/cn400012b (2013).

587 40 Akerboom, J. *et al.* Genetically encoded calcium indicators for multi-color neural activity imaging and
588   combination with optogenetics. *Frontiers in molecular neuroscience* **6**, 2 (2013).

589 41 Dana, H. *et al.* Sensitive red protein calcium indicators for imaging neural activity. *Elife* **5**,
590   doi:10.7554/eLife.12727 (2016).

591 42 Bungay, P. M., Newton-Vinson, P., Isele, W., Garris, P. A. & Justice Jr, J. B. Microdialysis of dopamine
592   interpreted with quantitative model incorporating probe implantation trauma. *Journal of neurochemistry*
593   **86**, 932-946 (2003).

594 43 Florin-Lechner, S. M., Druhan, J. P., Aston-Jones, G. & Valentino, R. J. Enhanced norepinephrine release
595   in prefrontal cortex with burst stimulation of the locus coeruleus. *Brain research* **742**, 89-97 (1996).

596 44 Pacak, K., Palkovits, M., Kopin, I. J. & Goldstein, D. S. Stress-induced norepinephrine release in the
597   hypothalamic paraventricular nucleus and pituitary-adrenocortical and sympathoadrenal activity: in vivo
598   microdialysis studies. *Frontiers in neuroendocrinology* **16**, 89-150 (1995).

599 45 Smith, A. D., Olson, R. J. & Justice, J. B., Jr. Quantitative microdialysis of dopamine in the striatum: effect
600   of circadian variation. *J Neurosci Methods* **44**, 33-41, doi:10.1016/0165-0270(92)90111-p (1992).

601 46 Wan, Q. *et al.* Mini G protein probes for active G protein–coupled receptors (GPCRs) in live cells. *Journal*
602   *of Biological Chemistry* **293**, 7466-7473 (2018).

603 47 Kroeze, W. K. *et al.* PRESTO-Tango as an open-source resource for interrogation of the druggable human
604   GPCRome. *Nature structural & molecular biology* **22**, 362 (2015).

605 48 Daw, N. D. & Tobler, P. N. in *Neuroeconomics*   283-298 (Elsevier, 2014).

606 49 Glimcher, P. W. Understanding dopamine and reinforcement learning: the dopamine reward prediction
607   error hypothesis. *Proceedings of the National Academy of Sciences* **108**, 15647-15654 (2011).

608 50 Gonzales, R. A., Job, M. O. & Doyon, W. M. The role of mesolimbic dopamine in the development and
609   maintenance of ethanol reinforcement. *Pharmacology & therapeutics* **103**, 121-146 (2004).

610 51 Broussard, G. J. *et al.* In vivo measurement of afferent activity with axon-specific calcium imaging. *Nat*
611   *Neurosci* **21**, 1272-1280, doi:10.1038/s41593-018-0211-4 (2018).

612 52 Schwaerzel, M. *et al.* Dopamine and octopamine differentiate between aversive and appetitive olfactory
613   memories in Drosophila. *Journal of Neuroscience* **23**, 10495-10502 (2003).

614 53 Kim, Y.-C., Lee, H.-G. & Han, K.-A. D1 dopamine receptor dDA1 is required in the mushroom body
615   neurons for aversive and appetitive learning in Drosophila. *Journal of Neuroscience* **27**, 7640-7647 (2007).

616 54 Schroll, C. *et al.* Light-induced activation of distinct modulatory neurons triggers appetitive or aversive
617   learning in Drosophila larvae. *Current biology* **16**, 1741-1747 (2006).

618 55 Claridge-Chang, A. *et al.* Writing memories with light-addressable reinforcement circuitry. *Cell* **139**, 405-
619   415 (2009).

620 56   Tanaka, N. K., Tanimoto, H. & Ito, K. Neuronal assemblies of the Drosophila mushroom body. *Journal of*
621   *Comparative Neurology* **508**, 711-755 (2008).

622 57   Mao, Z. & Davis, R. L. Eight different types of dopaminergic neurons innervate the Drosophila mushroom
623   body neuropil: anatomical and physiological heterogeneity. *Frontiers in neural circuits* **3**, 5 (2009).

624 58   Aso, Y. *et al.* The neuronal architecture of the mushroom body provides a logic for associative learning.
625   *elife* **3**, e04577 (2014).

626 59   Klapoetke, N. C. *et al.* Independent optical excitation of distinct neural populations. *Nature methods* **11**,
627   338 (2014).

628 60   Harada, K. *et al.* Red fluorescent protein-based cAMP indicator applicable to optogenetics and in vivo
629   imaging. *Scientific reports* **7**, 1-9 (2017).

630 61   Alcaro, A., Huber, R. & Panksepp, J. Behavioral functions of the mesolimbic dopaminergic system: an
631   affective neuroethological perspective. *Brain research reviews* **56**, 283-321 (2007).

632 62   Yizhar, O. *et al.* Neocortical excitation/inhibition balance in information processing and social dysfunction.
633   *Nature* **477**, 171-178 (2011).

634 63   Liang, X. *et al.* Morning and Evening Circadian Pacemakers Independently Drive Premotor Centers via a
635   Specific Dopamine Relay. *Neuron* **102**, 843-857 e844, doi:10.1016/j.neuron.2019.03.028 (2019).

636 64   Kim, H. R. *et al.* A unified framework for dopamine signals across timescales. *BioRixv*,
637   doi:10.1101/803437 (2019).

638 65   Jing, M. *et al.* A genetically encoded fluorescent acetylcholine indicator for in vitro and in vivo studies.
639   *Nat Biotechnol* **36**, 726-737, doi:10.1038/nbt.4184 (2018).

640 66   Jing, M. *et al.* An optimized acetylcholine sensor for monitoring in vivo cholinergic activity.
641   doi:10.1101/861690 (2019).

642 67   Feng, J. *et al.* A Genetically Encoded Fluorescent Sensor for Rapid and Specific In Vivo Detection of
643   Norepinephrine. *Neuron* **102**, 745-761 e748, doi:10.1016/j.neuron.2019.02.037 (2019).

644 68   Deng, B. *et al.* Chemoconnectomics: mapping chemical transmission in Drosophila. *Neuron* **101**, 876-893.
645   e874 (2019).

646 69   Gibson, D. G. *et al.* Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*
647   **6**, 343-345, doi:10.1038/nmeth.1318 (2009).

648 70   Yusa, K., Zhou, L., Li, M. A., Bradley, A. & Craig, N. L. A hyperactive piggyBac transposase for
649   mammalian applications. *Proceedings of the National Academy of Sciences* **108**, 1531-1536 (2011).

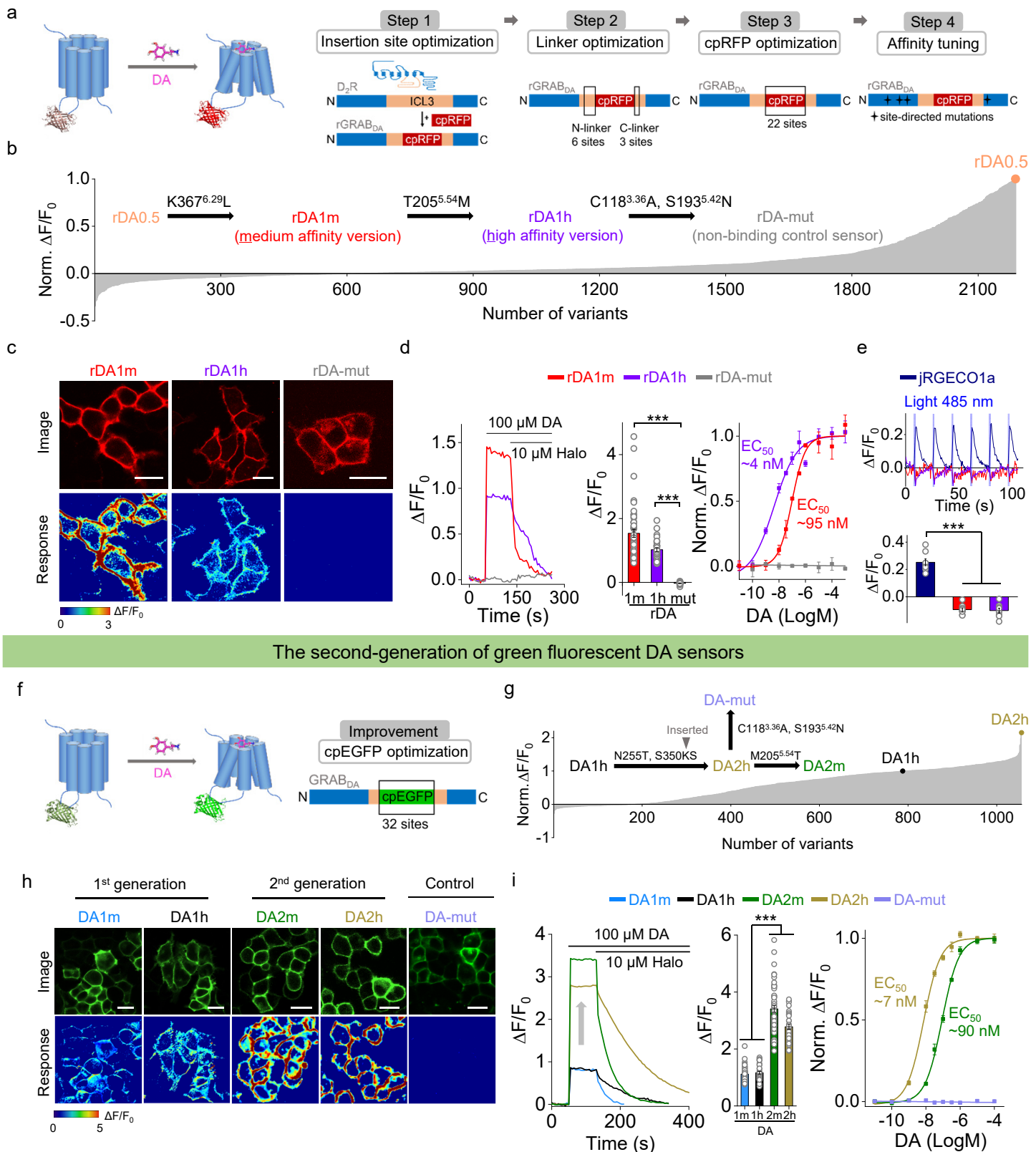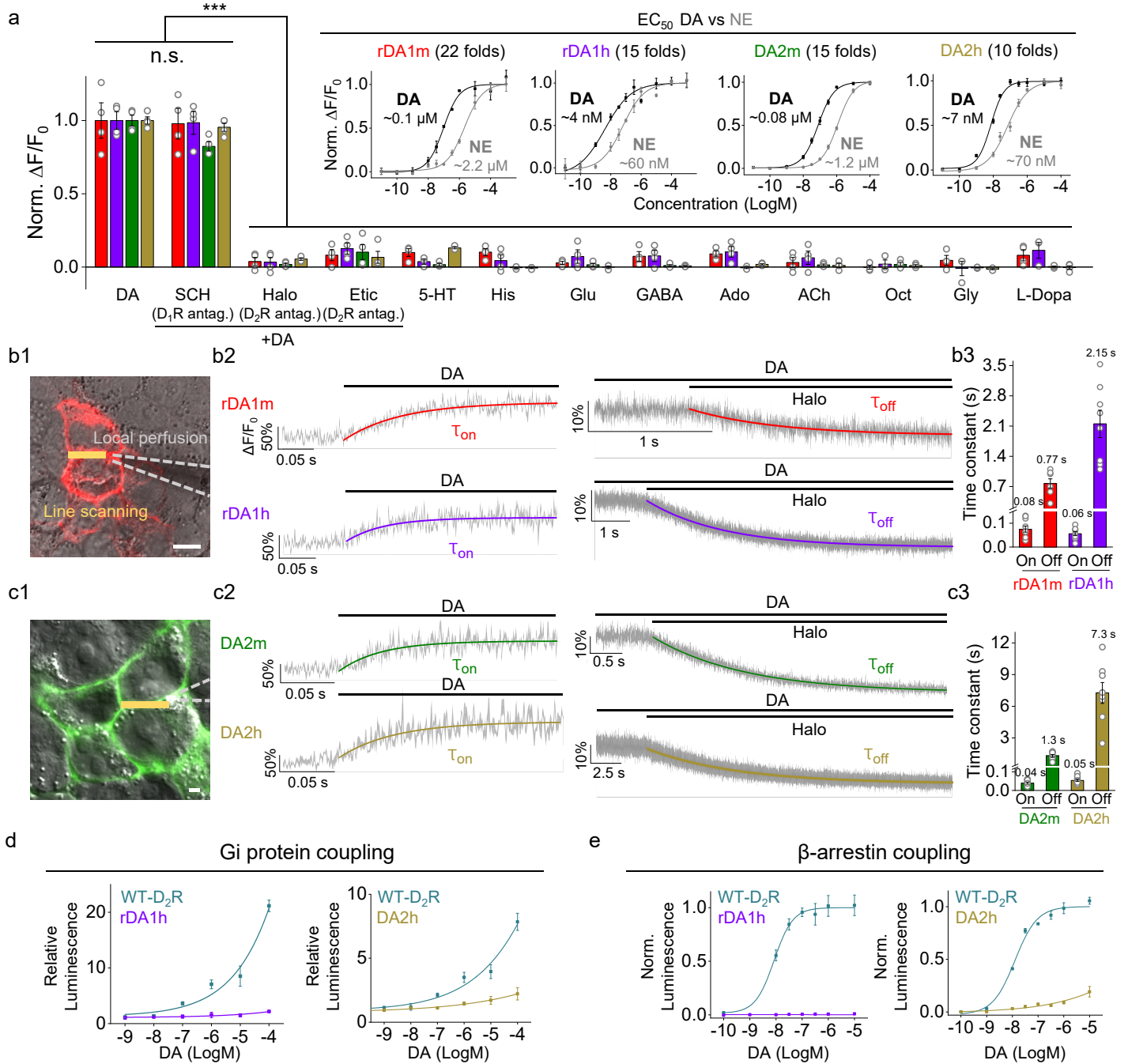650 71   Hull, E. M., Meisel, R. L. & Sachs, B. D. in *Hormones, brain and behavior*   3-137 (Elsevier, 2002).

651

Fig. 1 | Development and characterization of two novel red fluorescent DA sensors and second-generation green fluorescent DA sensors.

Fig. 2│ Characterization of GRAB_DA sensors in HEK293T cells and cultured neurons.

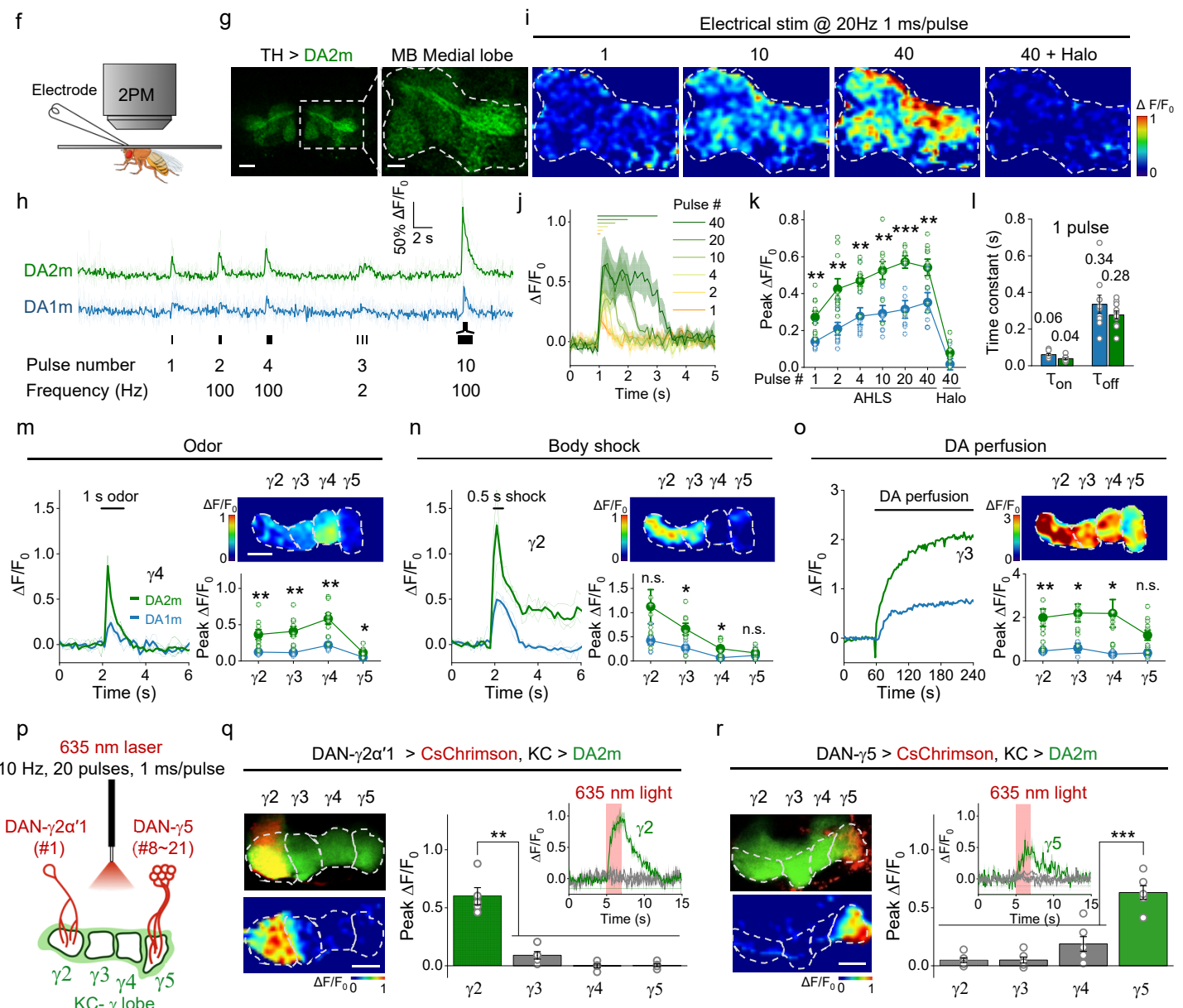**Fig. 3│ GRAB_DA sensors can be used to measure DA release in acute mouse brain slices.**
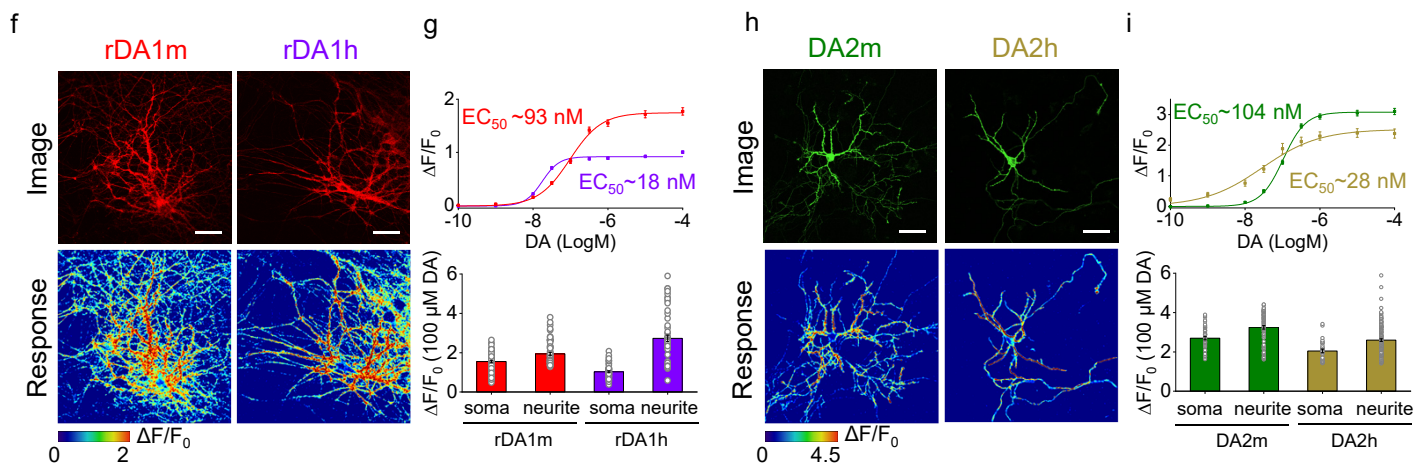
Fig. 4│ *In vivo* 2-photon imaging of DA dynamics in *Drosophila* using GRAB$_{DA}$ sensors.

Fig. 5 | GRAB$_{DA}$ sensors can detect optogenetically induced nigrostriatal DA release in freely moving mice.

Fig. 6 | GRAB$_{DA}$ sensors can be used to measure dopaminergic activity in the mouse NAc during sexual behavior.
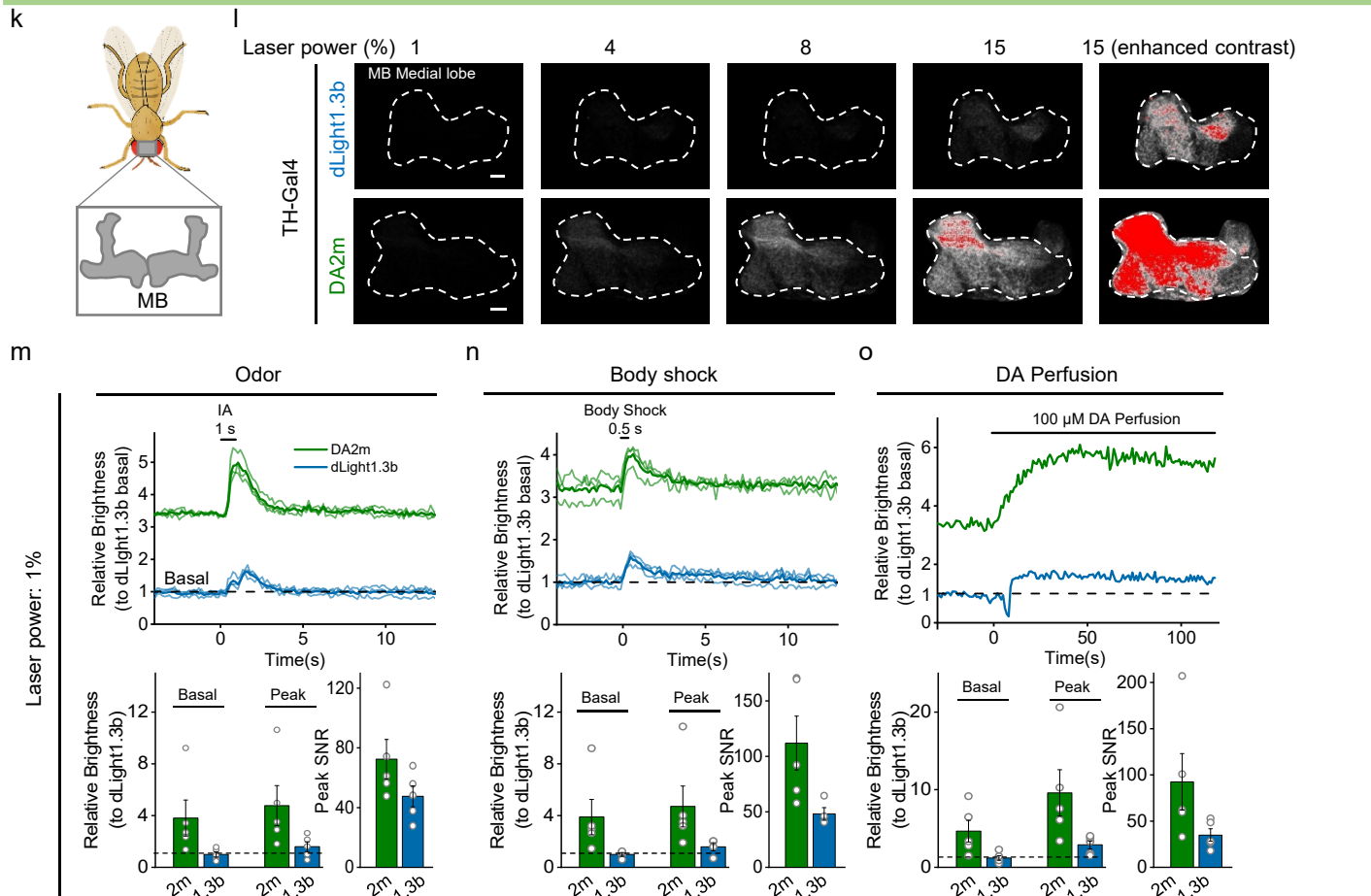
Supplementary Fig. S1│ Characterization of the sensors expressed in HEK293T cells (related to Fig. 2).
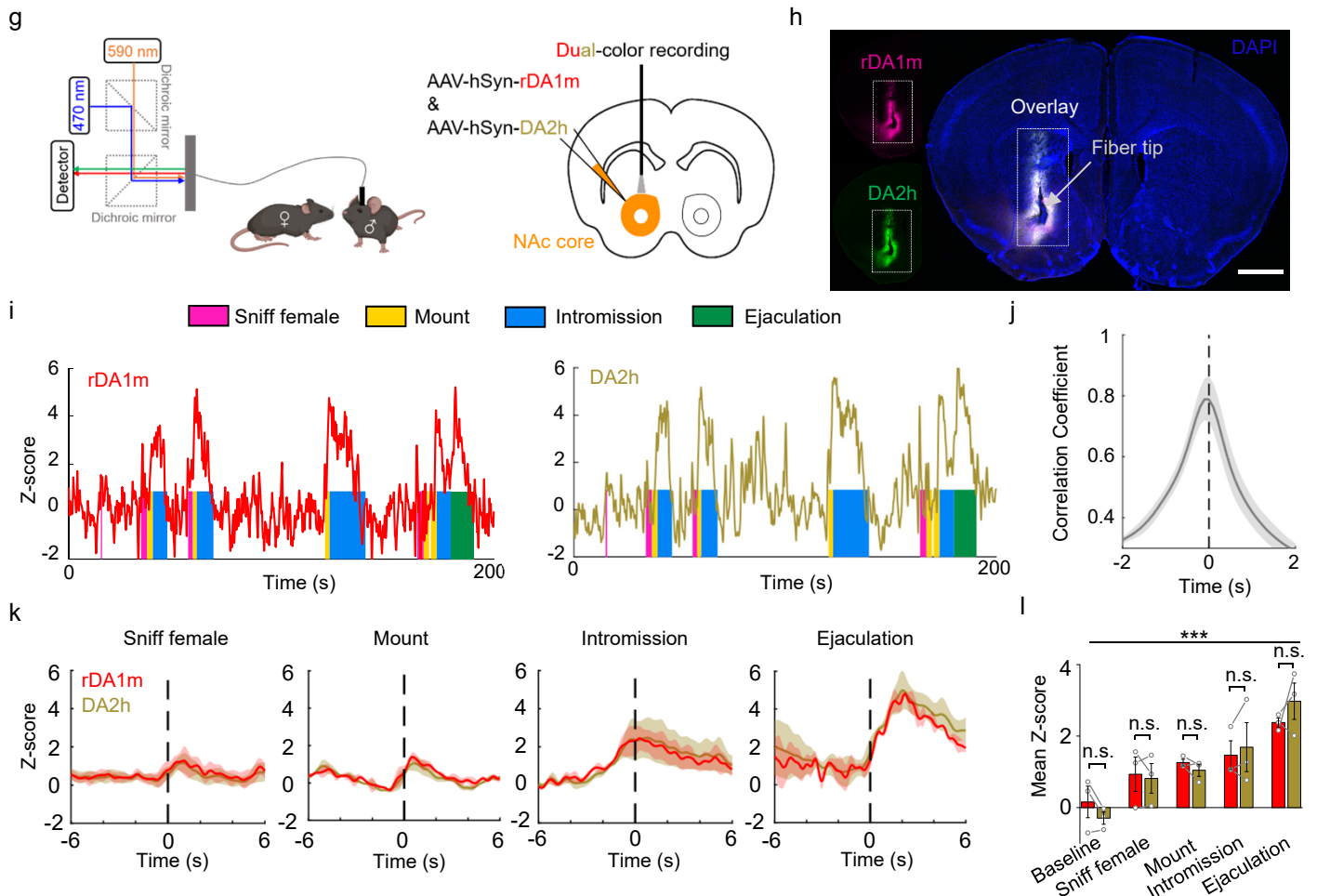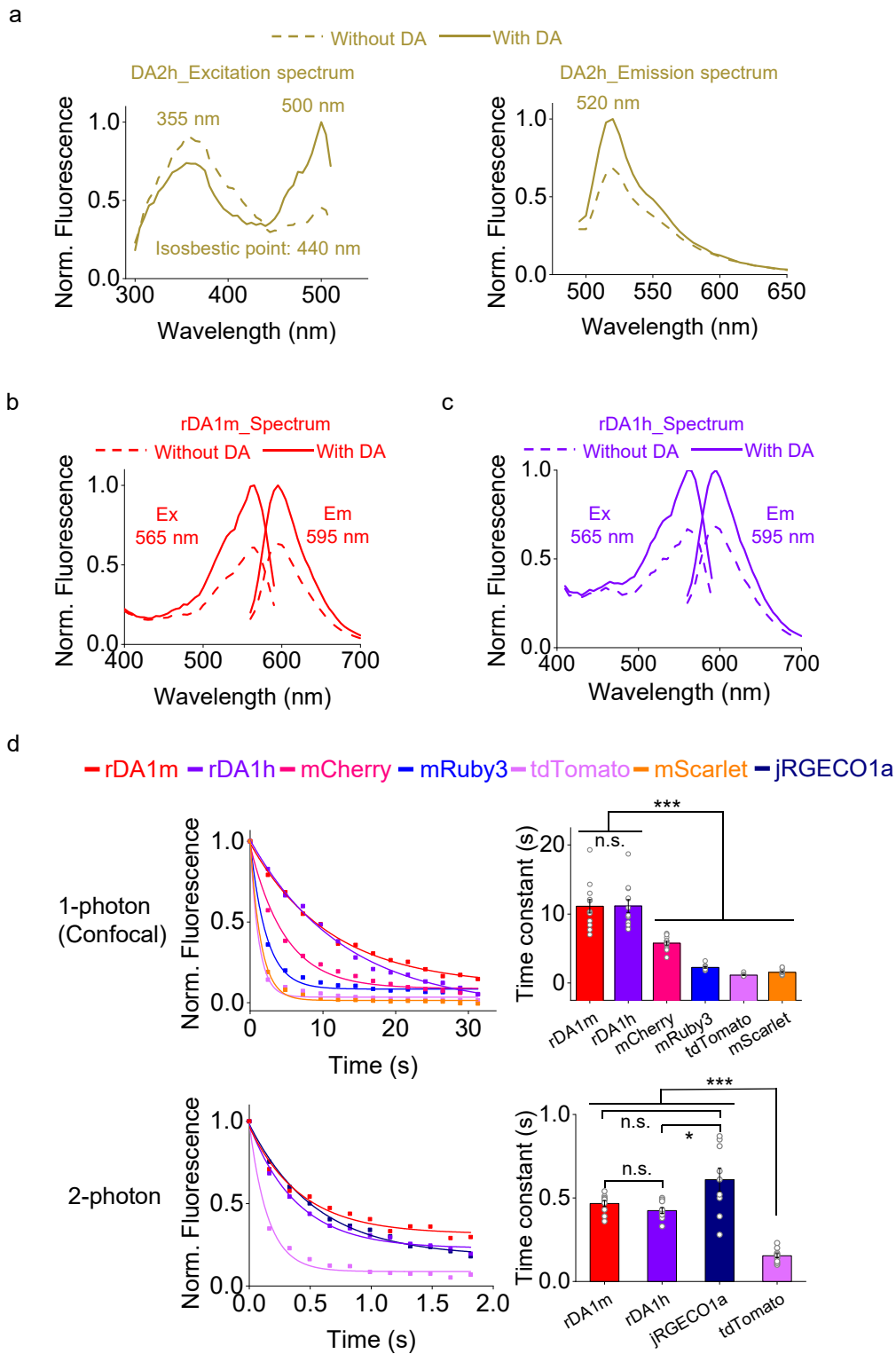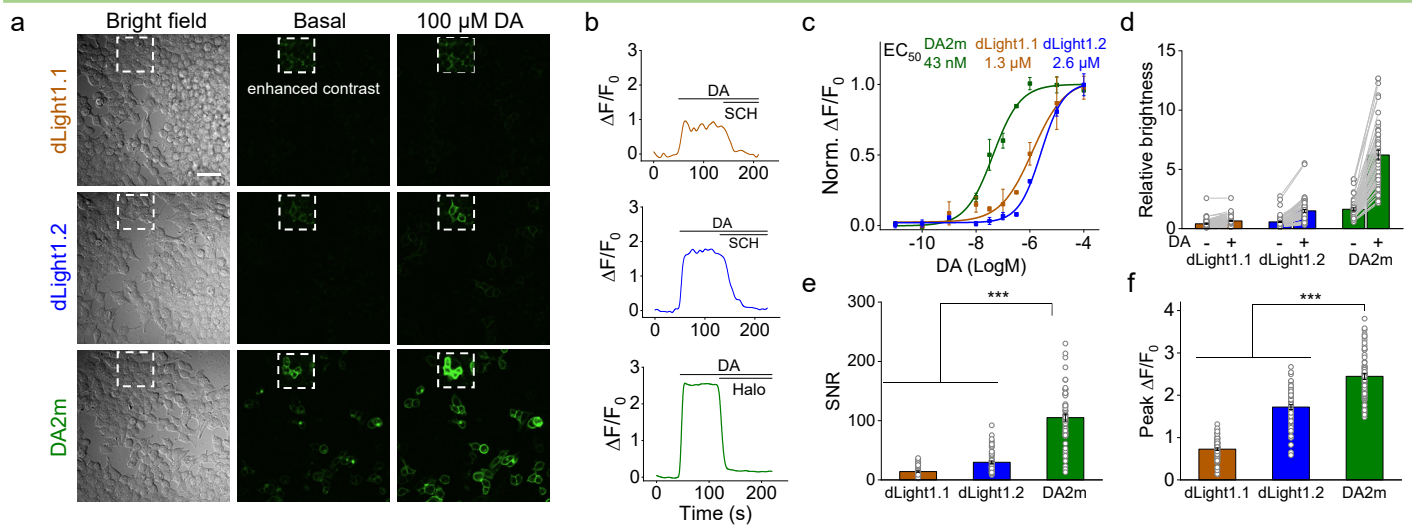
Supplementary Fig. S2│ Comparison between dLight and GRAB_DA (related to Figs. 2 and 4).
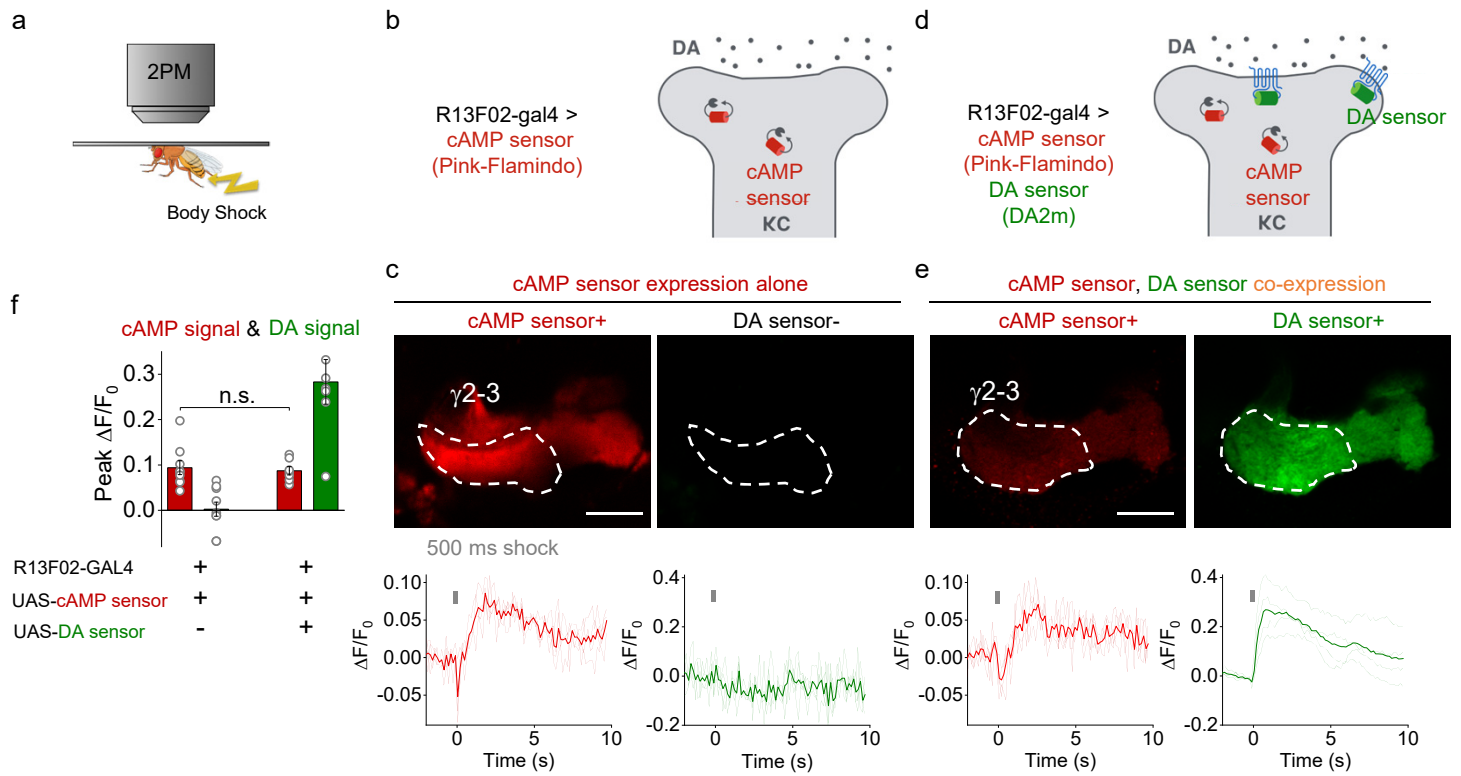
Supplementary Fig. S3 │ Expressing GRAB$_{DA2m}$ sensors does not affect cAMP signaling *in vivo* (related to Fig. 4).
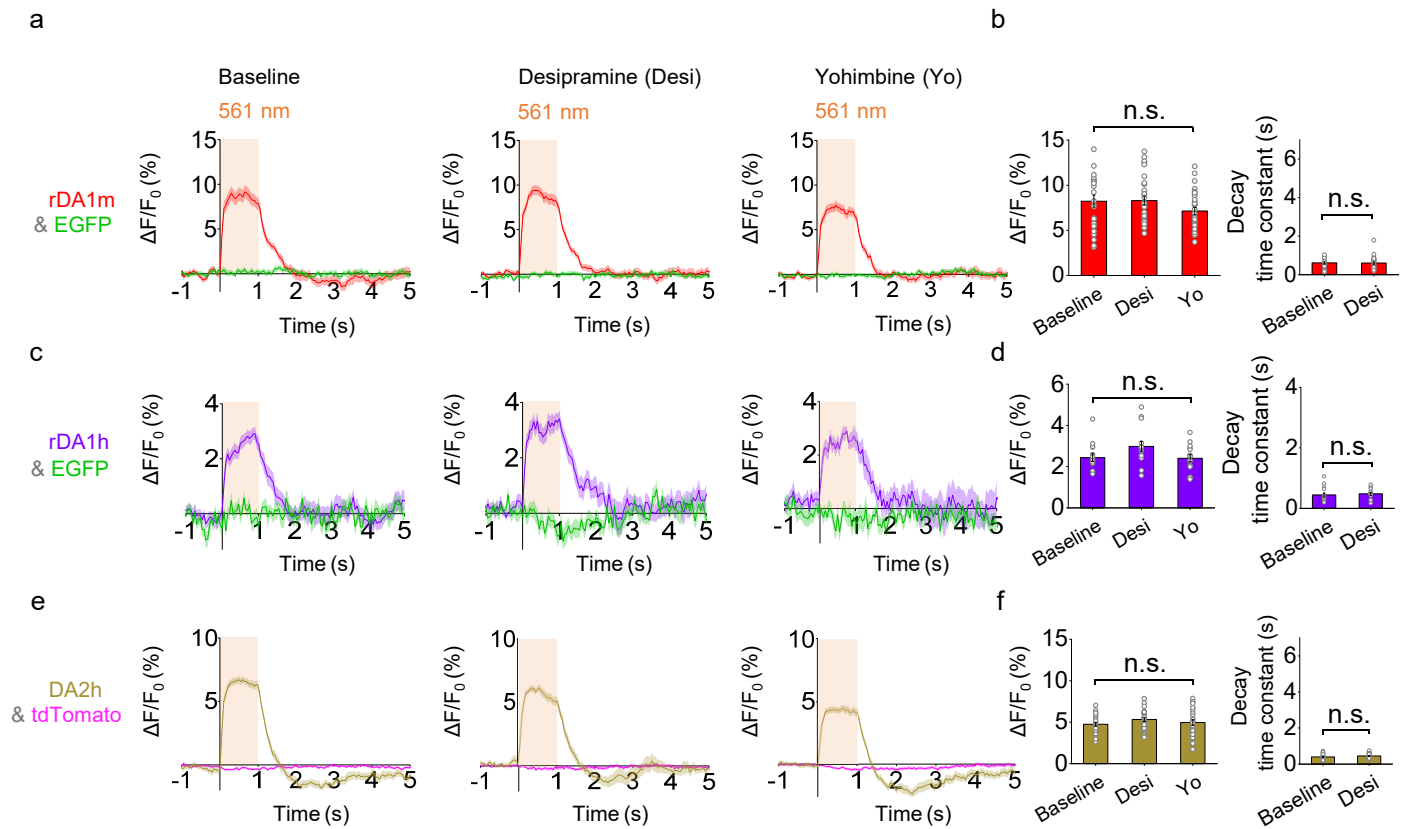
Supplementary Fig. S4 │ Optogenetically induced nigrostriatal DA release in freely moving mice is not affected by desipramine or yohimbine (related to Fig. 5).
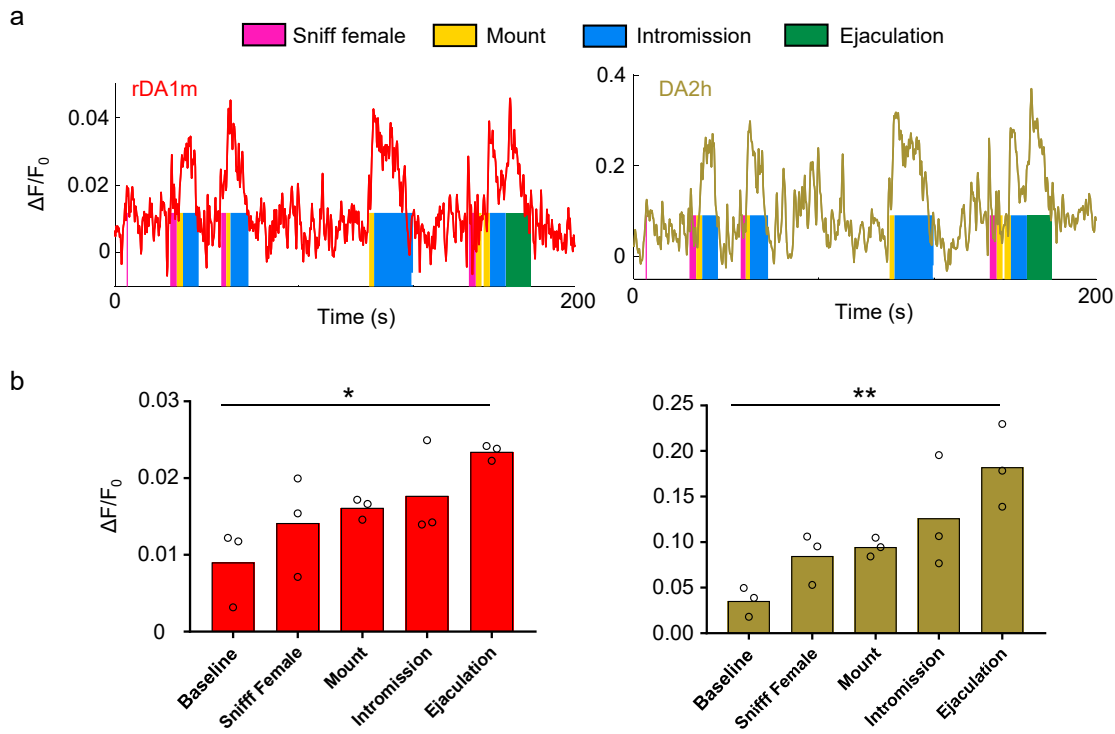
Supplementary Fig. S5│ The ΔF/F$_0$ signals of rDA1m and DA2h during sexual behavior (related to Fig. 6).
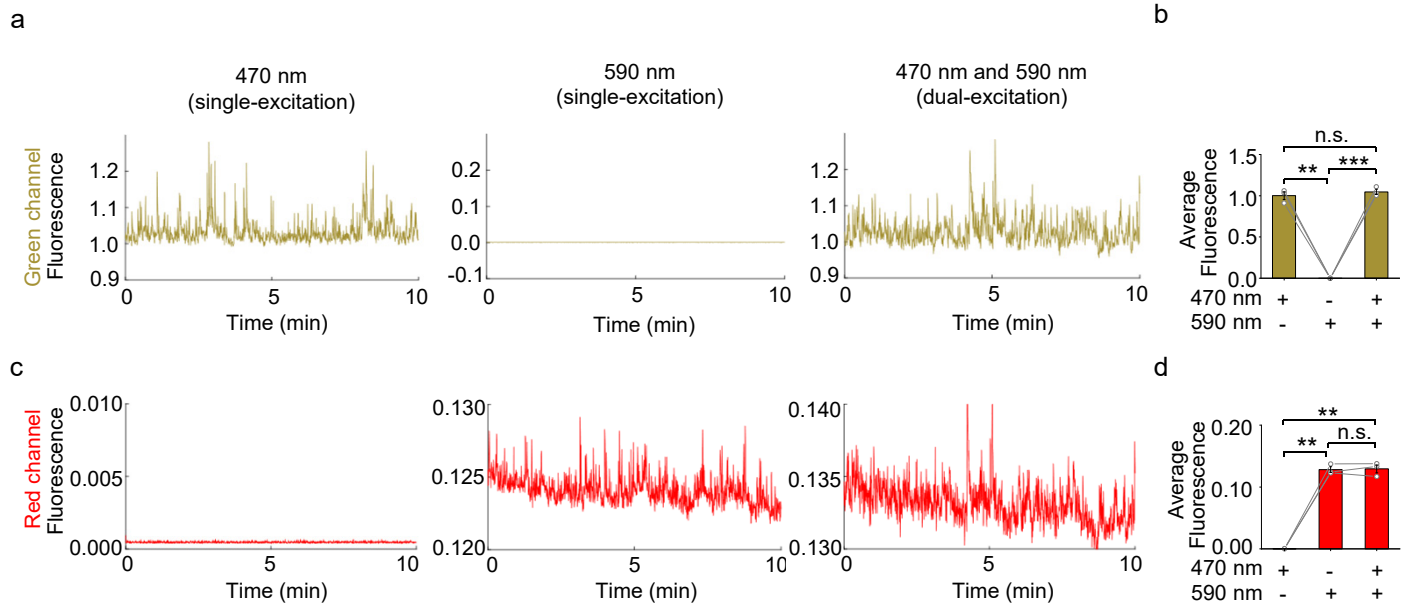
Supplementary Fig. S6│ The excitation wavelength for DA2h does not excite rDA1m, and *vice versa* (related to Fig. 6).

652 **Fig. 1 | Development and characterization of two novel red fluorescent DA sensors and**
653 **second-generation green fluorescent DA sensors.**

654 **a,f**, Schematic illustration showing the design and optimization of the red and green fluorescent
655 GRAB$_{DA}$ sensors.

656 **b**, Normalized change in fluorescence in response to 100 μM DA measured for red fluorescent DA
657 sensor variants during steps 1–3. A total of 2189 variants were screened, and the variant with the
658 highest fluorescence change (named rDA0.5) was selected for subsequent affinity tuning; rDA0.5
659 was then sequentially mutated as shown to generate rDA1m, rDA1h, and rDA-mut.

660 **c**, Representative red fluorescence images (top) and change in fluorescence in response to 100 μM
661 DA (bottom) in HEK293T cells expressing rDA1m, rDA1h or rDA-mut. Scale bars, 20 μm.

662 **d**, Representative traces (left), group summary of peak $\Delta F/F_0$ (middle; n=17–46 cells), and
663 normalized dose-response curves (right; n=3 wells with 200–400 cells/well) in response to DA.
664 Where indicated, 10 μM haloperidol (Halo) was applied.

665 **e**, Representative traces (top) and group summary (bottom; n=8–9 cells) of $\Delta F/F_0$ in response to
666 blue light in cells expressing jRGECO1a, rDA1m, or rDA1h.

667 **g**, Normalized $\Delta F/F_0$ in response to 100 μM DA application for 1056 variants, normalized to the
668 first-generation DA1h sensor. DA2h was then mutated as shown to generate DA2m and DA-mut.

669 **h**, Representative green fluorescence images (top) and change in fluorescence in response to 100
670 μM DA (bottom) in HEK293T cells expressing DA1m, DA1h, DA2m, DA2h, or DA-mut. Scale
671 bars, 20 μm.

672 **i**, Representative traces (left), group summary of peak $\Delta F/F_0$ (middle; n=33–68 cells), and
673 normalized dose-response curves (right; n=3 wells with 200–500 cells/well) in response to DA.
674 Where indicated, 10 μM Halo was applied.

675

676 **Fig. 2 | Characterization of GRAB$_{DA}$ sensors in HEK293T cells and cultured neurons.**

677 **a**, Normalized $\Delta F/F_0$ was measured in HEK293T cells expressing rDA1m, rDA1h, DA2m, or DA2h
678 following the application of DA alone, DA+SCH-23390, DA+haloperidol, DA+eticlopride,
679 serotonin (5-HT), histamine, glutamate, gamma-aminobutyric acid (GABA), adenosine,
680 acetylcholine (ACh), octopamine, glycine, or L-DOPA (all applied at 1 μM); n=3–4 wells with 200–
681 1200 cells/well. The insets show normalized dose-response curves for DA and norepinephrine (NE)
682 in cells expressing rDA1m, rDA1h, DA2m, or DA2h (n=3 wells with 200–800 cells/well).

683 **b-c**, Response kinetics for rDA1m, rDA1h, DA2m, and DA2h. **b1** and **c1**, Schematic illustration
684 showing the local perfusion system for applying compounds. DA and Halo were delivered via a
685 glass pipette (dashed gray lines) positioned at the sensor-expressing cells, and the fluorescence
686 signal was measured using confocal line scanning (thick yellow lines). Scale bars, 10 μm. **b2** and
687 **c2**, Representative traces showing fluorescence after application of DA (left) and subsequent
688 addition of Halo (right). The traces were the average of 3 different regions of interest (ROIs) on the
689 scanning line, shaded with ± s.e.m.. Each trace was fitted with a single-exponential function to
690 determine τ_{on} (left) and τ_{off} (right). **b3** and **c3**, Group summary of τ_{on} and τ_{off} (n=7–10 cells).

691 **d**, Gi coupling of wild-type (WT) D$_2$R, rDA1h, and DA2h was measured using the luciferase
692 complementation assay (n=3 wells each).

693 **e**, β-arrestin coupling of WT D$_2$R, rDA1h, and DA2h was measured using the TANGO assay (n=3
694 wells each).

695 **f,h**, Representative red (**f**) and green (**h**) fluorescence images (top) and response to 100 μM DA

696 (bottom) in neurons expressing the indicated sensors. Scale bars, 10 μm.

697 **g,i**, Dose-response curves (top) and group summary (bottom) of the responses measured in the soma

698 and neurites of neurons expressing the indicated sensors (n=21–60 neurons).

699

700 **Fig. 3 ┃ GRAB$_{DA}$ sensors can be used to measure DA release in acute mouse brain slices.**

701 **a**, Schematic illustration depicting the experimental design for injecting AAVs expressing the

702 rDA1m, rDA1h, or DA2m sensors in the mouse NAc, followed by imaging of electrical stimulation–

703 evoked DA release in the NAc in acute brain slices.

704 **b**, Representative fluorescence images showing the expression of rDA1m, rDA1h, and DA2m in

705 the NAc. The anterior cingulate area (aca) adjacent to the NAc is indicated. Scale bar, 100 μm.

706 **c**, Fluorescence images measured in brain slices from mice expressing rDA1m, rDA1h, or DA2m

707 in the NAc. The dashed circles indicate the ROIs used to analyze the signals.

708 **d**, Representative traces showing the normalized change in fluorescence (top) and group summary

709 of τ_{on} and τ_{off} (bottom) for rDA1m, rDA1h, and DA2m in response to 10 electrical stimuli applied

710 at 100 Hz (n=3–5 slices from 2–3 mice). The data were processed with 2× binning.

711 **e**, Representative traces and group summary of the change in fluorescence measured for rDA1m

712 (left), rDA1h (middle), and DA2m (right) in response to the indicated number of electrical stimuli

713 applied at 20 Hz; where indicated, Halo (10 μM) was included in the bath solution (n=3–7 slices

714 from 2–4 mice).

715 **f**, Schematic illustration depicting the strategy for injecting AAVs in the NAc and VTA, followed

716 by simultaneous recording of Ca^{2+} and DA release using dual-color imaging.

717 **g**, Representative green fluorescence (GCaMP6s), red fluorescence (rDA1m), and merged images

718 of the NAc. The aca adjacent to the NAc is indicated. Scale bar, 100 μm.

719 **h**, Fluorescence images showing the response of axon-GCaMP6s and rDA1m following 20

720 electrical stimuli applied at 20 Hz. The dashed circles indicate the ROIs used to analyze the signals.

721 **i-j**, Representative traces (**i**) and group summary (**j**) of the change in axon-GCaMP6s and rDA1m

722 fluorescence in response to 20 electrical stimuli applied at 20 Hz in the absence or presence of Halo

723 (10 μM); n=3 slices from 3 mice.

724 **k**, The peak change in rDA1m fluorescence plotted against the peak change in axon-GCaMP6s

725 fluorescence in response to various numbers of pulses applied at 20 Hz. The data were fitted to a

726 linear function, and the coefficient of correlation is shown (n=8 slices from 3 mice).

727 Average traces shaded with ± s.e.m from one slice are shown for representation.

728

729 **Fig. 4 ┃ *In vivo* 2-photon imaging of DA dynamics in *Drosophila* using GRAB$_{DA}$ sensors.**

730 **a**, Schematic illustration depicting the experimental setup for imaging fluorescence changes in

731 *Drosophila* expressing GRAB$_{DA}$ sensors using 2-photon microscopy (2PM). Application of various

732 stimuli are also depicted.

733 **b**, Representative red fluorescence images of a transgenic fly expressing rDA1m in the Kenyon cells

734 (KCs), with an expanded view of the olfactory mushroom body (MB) medial lobe. Scale bars, 20

735 μm (top) and 10 μm (bottom).

736 **c-e**, Representative images (top; the dashed area indicates the MB medial lobe), traces (middle), and

737 group summary (bottom) of $\Delta F/F_0$ in response to a 1 s application of odorant (**c**), a 500 ms body

738 shock (**d**), and 100 μM DA (**e**) in flies expressing rDA1m in saline, rDA1m in Halo, or rDA1m-mut

739 in saline (n=5–15 flies each).

f, Schematic illustration depicting the experimental setup for imaging electrical stimulation–evoked DA release.

g, Representative green fluorescence images of a transgenic fly expressing DA2m in dopaminergic neurons (DANs), with an expanded view of the MB medial lobe. Scale bars, 20 μm (left) and 10 μm (right).

h, Representative traces of DA2m and DA1m fluorescence; where indicated, electrical stimuli were applied.

i,j, Representative images (**i**, the dashed area indicates the MB medial lobe) and traces (**j**) of DA2m $\Delta F/F_0$ in response to electrical stimuli applied at 20 Hz in the absence or presence of Halo.

k, Group summary of DA2m and DA1m $\Delta F/F_0$ in response to electrical stimuli applied at 20 Hz in the absence or presence of Halo (n=9–10 flies each).

l, Kinetics (τ_{on} and τ_{off}) of DA2m and DA1m in response to a single electrical stimulus (n=9–10 flies each).

m-o, Representative traces (left), fluorescence images (upper right), and group summary (bottom right) of the indicated MB lobe compartments in response to a 1 s application of odorant (**m**), a 500 ms body shock (**n**), and 100 μM DA (**o**); n=3–10 flies each.

p, Schematic illustration depicting the strategy for imaging optogenetically induced DA release. DA2m is expressed in the KCs, and CsChrimson is expressed in the DANs in either the γ2 or γ5 MB compartment (with the number of innervating cells indicated).

q,r, Representative fluorescence images of CsChrimson (upper left), DA2m (lower left), representative traces (top right), and group summary (bottom right) of DA2m fluorescence in the γ2 (**q**) and γ5 (**r**) MB compartments in response to optogenetic stimulation (n=5–6 flies each). Scale bars, 20 μm.

The group data for DA1m shown in panels **k**, **m**, **n**, and **o** were reproduced from Sun et al.[31] with permission. Average traces (bold), overlaid with single-trial traces (light) or shaded with ± s.e.m, from one fly are shown for representation.

Fig. 5┃ GRAB$_{DA}$ sensors can detect optogenetically induced nigrostriatal DA release in freely moving mice.

a, Schematic illustration depicting the experimental setup for dual-color fiber photometry recording in the dorsal striatum while optogenetically stimulating DANs in the SNc. The combinations of sensor and fluorescent protein were used in the experiments shown in panels **c-l**.

b, Measured emission spectra of rDA1m and EGFP (left) and DA2m and tdTomato (right).

c,e,g,i,k, Average $\Delta F/F_0$ traces of the indicated sensors and fluorescent proteins during optogenetic stimulation under control conditions (left) or in the presence of methylphenidate (MPH) or eticlopride (Etic).

d,f,h,j,l, Group summary of $\Delta F/F_0$ and τ_{off} (where applicable) for the corresponding sensors in panels **c**, **e**, **g**, **i**, and **k**, respectively (n=15–30 trials from 3–6 hemispheres of 3–4 mice per condition).

Fig. 6┃ GRAB$_{DA}$ sensors can be used to measure dopaminergic activity in the mouse NAc during sexual behavior.

a, Schematic illustration depicting the strategy for injecting AAVs and performing bilateral fiber photometry recording.

b, Representative image showing the expression of DA1h and DA2h in opposite hemispheres, as

784   well as the recording site ('fiber tip'). The nuclei were counterstained with DAPI (blue). Scale bar,
785   1 mm.
786   **c**, Representative traces of DA1h and DA2h $\Delta F/F_0$ measured during the indicated stages of mating.
787   **d**, Summary of the time shift correlation coefficient between the DA1h and DA2h signals (n=3
788   mice).
789   **e**, Average post-stimulus histograms showing the $\Delta F/F_0$ signals of DA1h and DA2h aligned to the
790   onset of the indicated mating events (n=3 mice).
791   **f**, Group summary of mean $\Delta F/F_0$ measured for DA1h and DA2h during the indicated mating events
792   (n=3 mice).
793   **g**, Schematic illustration depicting the strategy for injecting AAVs and performing dual-color fiber
794   photometry recording.
795   **h**, Representative images showing the colocalized expression of rDA1m (red) and DA2h (green),
796   as well as the recording site. The nuclei were counterstained with DAPI (blue). Scale bar, 1 mm.
797   **i**, Representative traces of the concurrent Z-score signals of rDA1m (left) and DA2h (right) during
798   the indicated stages of sexual behavior.
799   **j**, Summary of the time shift correlation coefficient between the rDA1m and DA2h signals (n=3
800   mice).
801   **k**, Average post-stimulus histograms showing the Z-score signals of rDA1m and DA2h aligned to
802   the onset of the indicated mating events (n=3 mice).
803   **l**, Group summary of the mean Z-scores measured for rDA1m and DA2h during the indicated mating
804   events (n=3 mice).
805
806   **Supplementary Fig. S1┃ Characterization of the sensors expressed in HEK293T cells (related**
807   **to Fig. 2).**
808   **a-c**, The excitation and emission spectra of DA2h (**a**), rDA1m (**b**), and rDA1h (**c**) in the absence
809   and presence of DA.
810   **d**, The photostability of rDA1m and rDA1h (in the presence of 100 μM DA) and the indicated
811   fluorescent proteins was measured using 1-photon (top) and 2-photon (bottom) microscopy. Left,
812   representative photobleaching curves; each curve was fitted to a single-exponential function. Right,
813   group summary of the photobleaching time constants (n=10–12 cells each).
814
815   **Supplementary Fig. S2┃ Comparison between dLight and GRAB_{DA} (related to Figs. 2 and 4).**
816   **a**, Representative bright-field and fluorescence images measured before (baseline) and after
817   application of DA in HEK293T cells expressing dLight1.1, dLight1.2, or DA2m. Scale bar, 50 μm.
818   **b**, Representative traces of dLight1.1, dLight1.2, and DA2m $\Delta F/F_0$ in response to 100 μM DA
819   followed by either 10 μM SCH (dLight1.1 and dLight1.2) or 10 μM Halo (DA2m).
820   **c**, Normalized dose-response curves for dLight1.1, dLight1.2, and DA2m (n=3 wells with 100–500
821   cells/well).
822   **d-f**, Group summary of the relative brightness (**d**), signal-to-noise ratio (SNR) (**e**), and peak $\Delta F/F_0$
823   (**f**) for dLight1.1, dLight1.2, and DA2m in response to 100 μM DA (n=35–77 cells each). To assess
824   brightness, the membrane–targeted red fluorescent protein mCherry-CAAX was co-expressed with
825   each sensor, and relative brightness was normalized to the corresponding mCherry signal.
826   **g-j**, Similar to **a-f**, except that dLight1.1 and DA2m were expressed in cultured neurons (n=28–30
827   neurons each). Scale bar, 50 μm.

828 **k**, Schematic illustration depicting the location of the *Drosophila* olfactory mushroom body (MB).

829 **l**, Fluorescence images of the MB in flies expressing dLight1.3b (top) or DA2m (bottom) using 2-

830 photon microscopy at the indicated laser power settings. Enhanced-contrast images at 15% laser

831 power are shown at the right. Fluorescence is shown in grayscale, with saturated pixels shown in

832 red. Scale bars, 10 μm.

833 **m-o**, Representative traces (top) and group summary of relative brightness measured for dLight1.3b

834 and DA2m during odorant application (**m**), body shock (**n**), and DA perfusion (**o**); n=4–5 flies each.

835 Average traces (bold) overlaid with single-trial traces (light) from one fly are shown for

836 representation.

837

838 **Supplementary Fig. S3 ┃ Expressing GRAB$_{DA2m}$ sensors does not affect cAMP signaling *in***

839 ***vivo* (related to Fig. 4).**

840 **a**, Schematic illustration depicting the experimental setup for imaging body shock–evoked DA

841 release using 2-photon microscopy.

842 **b-e**, Schematic illustrations depicting the experimental strategy (**b,d**) and representative

843 fluorescence images and $\Delta F/F_0$ traces (**c,e**) in flies expressing the cAMP sensor Pink-Flamindo (**b,c**)

844 or co-expressing Pink-Flamindo and DA2m (**d,e**) in MB KCs. Where indicated, a 500 ms body

845 shock was delivered. The ROIs for measuring the γ2-γ3 compartments in the MB are indicated by

846 dashed white lines. Scale bars, 25 μm. Average traces (bold) overlaid with single-trial traces (light)

847 from one fly are shown for representation.

848 **f**, Group summary of peak Pink-Flamindo and DA2m $\Delta F/F_0$ measured under the indicated

849 conditions (n=7–9 flies each).

850

851 **Supplementary Fig. S4 ┃ Optogenetically induced nigrostriatal DA release in freely moving**

852 **mice is not affected by desipramine or yohimbine (related to Fig. 5).**

853 **a,c,e**, Average traces of $\Delta F/F_0$ in mice expressing rDA1m and EGFP (**a**), rDA1h and EGFP (**c**), or

854 DA2h and tdTomato (**e**) in the dorsal striatum. Where indicated, the experiments were conducted in

855 mice treated with either the norepinephrine transporter blocker desipramine or the $\alpha_{2A}R$ antagonist

856 yohimbine.

857 **b,d,f**, Group summary of $\Delta F/F_0$ and τ_{off} for the experiments shown in **a**, **c**, and **e**, respectively (n=25–

858 30 trials from 5–6 hemispheres of 3–6 mice each).

859

860 **Supplementary Fig. S5 ┃ The $\Delta F/F_0$ signals of rDA1m and DA2h during sexual behavior**

861 **(related to Fig. 6).**

862 **a**, Representative traces of rDA1m and DA2h $\Delta F/F_0$ measured during the indicated stages of mating.

863 **b**, Group summary of the $\Delta F/F_0$ measured for rDA1m and DA2h during the indicated mating events

864 (n=3 mice).

865

866 **Supplementary Fig. S6 ┃ The excitation wavelength for DA2h does not excite rDA1m, and**

867 ***vice versa* (related to Fig. 6).**

868 Mice co-expressing DA2h and rDA1m in the NAc were used for these experiments. Delivery of 470

869 nm light excited DA2h (green) but had no effect on rDA1m (red); conversely, delivery of 590 nm

870 light excited rDA1m but had no effect on DA2h. Delivery of both 470 nm and 590 nm light using

871 dual-wavelength excitation excited both DA2h and rDA1m. Shown at the right are the group

872   summaries (n=3 mice each).

873