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3	Improved dual-color GRAB sensors for monitoring dopaminergic activity in vivo
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#### 30 Abstract

Dopamine (DA) plays multiple roles in a wide range of physiological and pathological processes via a vast network 31 32 of dopaminergic projections. To fully dissect the spatiotemporal dynamics of DA release in both dense and sparsely 33 innervated brain regions, we developed a series of green and red fluorescent GPCR activation-based DA (GRAB<sub>DA</sub>) 34 sensors using a variety of DA receptor subtypes. These sensors have high sensitivity, selectivity, and signal-to-noise 35 properties with subsecond response kinetics and the ability to detect a wide range of DA concentrations. We then used these sensors in freely moving mice to measure both optogenetically evoked and behaviorally relevant DA 36 37 release while measuring neurochemical signaling in the nucleus accumbens, amygdala, and cortex. Using these 38 sensors, we also detected spatially resolved heterogeneous cortical DA release in mice performing various behaviors. These next-generation GRAB<sub>DA</sub> sensors provide a robust set of tools for imaging dopaminergic activity under a 39 40 variety of physiological and pathological conditions.

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#### 42 **Main**

43 Dopamine (DA) is a physiologically essential monoamine neuromodulator released by dopaminergic neurons that 44 project throughout the central nervous system. Interestingly, high spatial heterogeneity in terms of dopaminergic innervation—and therefore DA release—has been reported in various brain regions<sup>1–3</sup>. The DA system is known for 45 its roles in reward and reinforcement learning, motor function<sup>3,4</sup>, memory consolidation<sup>5,6</sup>, and emotional control<sup>7</sup>. 46 These processes are mediated by dopaminergic circuits originating from the midbrain projecting to the striatum and 47 48 nucleus accumbens (NAc), with the dorsal striatum and NAc receiving dense dopaminergic innervation. In contrast, the medial prefrontal cortex (mPFC) and amygdala receive relatively sparse dopaminergic innervation that regulates 49 a wide range of brain functions important in mediating cognitive function<sup>8,9</sup>, social interactions<sup>10</sup>, and aversive 50 sensing<sup>11,12</sup>. However, due to limitations in our ability to detect DA with high sensitivity and resolution, the 51 52 spatiotemporal dynamics of dopaminergic transmission in these sparsely innervated brain regions remain largely 53 unknown, particularly under various in vivo conditions. The ability to directly visualize and compare the dynamics 54 of DA release in both densely and sparsely innervated regions under behaviorally relevant conditions will therefore 55 provide valuable information regarding the spatiotemporal regulation of dopaminergic activity in the brain. However, 56 measuring DA with high sensitivity in order to understand how dopaminergic signaling is affected by neuronal activity and/or other neuromodulators requires multiplexed DA imaging combined with optogenetics and the 57 simultaneous imaging of other neurochemical processes. 58

59 Recent advances in the development of genetically encoded fluorescent sensors for detecting DA have led to 60 robust tools that can measure dopaminergic signals at high spatial and temporal resolution. By combining a G 61 protein-coupled receptor (GPCR) with circularly permutated fluorescent protein (cpFPs), our group and Tian's group 62 developed a series of genetically encoded green and red fluorescent DA sensors called GRAB<sub>DA</sub> and dLight<sup>13–16</sup>, which can be used to measure DA release under physiological and pathological conditions with high spatiotemporal 63 resolution. These early generation of sensors enabled us to expand the knowledge of spatiotemporal dynamics of 64 DA transmission in reward, learning and movement<sup>17–20</sup>, in strongly innervated regions such as striatum where 65 dopamine levels are high. However, tracking slight changes in DA levels in vivo, including tonic DA release and 66 67 fluctuations in sparsely innervated regions, was less possible because of limitations in sensitivity, especially with 68 bulk measurements like photometry. In addition, even in dense areas, better sensors with improved sensitivity and 69 signal-to-noise ratio are required for DA imaging with higher spatiotemporal precision. Moreover, the performance 70 of previously developed red-shifted DA sensors is relatively poor compared to green fluorescent sensors, greatly limiting their use. To overcome these issues, we performed large-scale rational mutagenesis and cell-based 71 72 screening in order to develop a next-generation series of red and green fluorescent GRAB<sub>DA</sub> sensors with extremely 73 high sensitivity, a high signal-to-noise ratio, and a wider concentration detection range for measuring DA release in 74 a wide range of brain regions.

#### 75 **RESULTS**

Engineering and characterization of DA sensors in cultured cells. To obtain next-generation DA sensors with 76 77 improved sensitivity, selectivity, and distinct pharmacology profiles, we used various DA receptor subtypes cloned 78 from several species as the sensor scaffold and replaced each receptor's third intracellular loop (ICL3) with the ICL3 previously used in existing GRAB sensors<sup>13,15,16,21</sup>. We identified several chimera prototypes with promising 79 performances in the initial screening by transplanting the ICL3 of existing sensors to different sites dopamine 80 receptors, for example, transplanting the ICL3 of rGRAB<sub>DA</sub> to Solenopsis invicta dopamine D2-like receptor (hereafter 81 termed red fire ant  $D_2R$ ) and the ICL3 of  $GRAB_{NE}$  to bovine  $D_1R$ . Interestingly, we also obtained good candidates 82 when re-engineering the ICL3s of dLight1.3b<sup>13</sup> and RdLight1<sup>15</sup> to their original GPCR backbone, i.e., the human D<sub>1</sub>R 83 (Fig. 1a; Extended Data Figs. 1 and 2). We then systematically optimized the length and amino acid composition of 84 85 the linker sequences, key residues in the cpFP that affect protein folding and/or fluorescence intensity<sup>22–27</sup>, and sites in the GPCR that affect ligand binding and/or structural coupling<sup>28–31</sup>, and screened a total of 5000 variants 86 (Extended Data Figs. 1 and 2). Using maximum brightness and the DA-induced change in fluorescence as our 87 88 selection criteria, our screening yielded a series of top-performing DA sensors with various DA receptor backbones, 89 including the green fluorescent gDA3m (based on human  $D_1R$ ) and gDA3h (based on bovine  $D_1R$ ) sensors, the red-90 shifted rDA2m and rDA2h (based on red fire ant D<sub>2</sub>R) sensors, and the red-shifted rDA3m and rDA3h (based on human D<sub>1</sub>R) sensors (Fig. 1b and 1c; Extended Data Fig. 4a), with "m" and "h" referring to medium and high DA 91 92 affinity, respectively. We also generated DA-insensitive versions of these sensors by introducing mutations in the ligand-binding pocket of the corresponding GPCRs, yielding gDA3mut, rDA2mut, and rDA3mut for use as negative 93 94 controls (Extended Data Figs. 1-3).

95 All six of our newly generated GRAB<sub>DA</sub> sensors localized well to the cell membrane when expressed in HEK293T 96 cells and exhibited a large increase in fluorescence in response to bath application of 100 µM DA (Fig. 1d); moreover, 97 the mutant versions were expressed at the cell surface but failed to respond to DA application (Extended Data Fig. 98 3). The sensors' affinities were within physiological DA levels and ranged from nanomolar to submicromolar 99 concentrations, with EC<sub>50</sub> values of 22 nM and 86 nM for gDA3h and gDA3m, respectively, and 9 nM, 180 nM, 20 nM, and 130 nM for rDA2h, rDA2m, rDA3h, and rDA3m, respectively (Fig. 1e and Supplementary Table 1). We also 100 101 compared these new sensors' performance with previously reported GRAB<sub>DA</sub> (gDA2m and rDA1m) and dLight (dLight1.3b and RdLight1) sensors<sup>13–16</sup> in cultured cells. With respect to the green fluorescent sensors, both gDA3m 102 and gDA3h had a >2-fold larger increase in fluorescence (with peak  $\Delta F/F_0$  values of ~1000%) and a higher signal-to-103 noise ratio (SNR) compared to gDA2m and dLight1.3b (Fig. 1f-h and Supplementary Table 1). With respect to the 104 red-shifted sensors, relative to rDA1m the basal fluorescence intensity values were 152%, 282%, 30%, 33%, and 16% 105 for rDA2m, rDA2h, rDA3m, rDA3h, and RdLight1, respectively; moreover, rDA2m and rDA2h had the largest dynamic 106 range (with peak  $\Delta F/F_0$  values of ~560% and ~240%, respectively) among the D<sub>2</sub>R-based red-shifted sensors (Fig. 1f-107 108 h and Supplementary Table 1). Finally, rDA3m and rDA3h had significantly higher brightness levels, fluorescence responses (with  $\Delta F/F_0$  values of ~1000%), and SNR compared to RdLight1 (Fig. 1f-h). These results suggest that these 109 next-generation GRAB<sub>DA</sub> sensors might be useful for imaging DA release in vivo both in DA-abundant conditions and 110 in brain regions with sparse dopaminergic innervation. 111

Next, we examined the properties of our new sensors when expressed in cultured neurons. Consistent with our results obtained using HEK293T cells, we found that the sensors localized well to the neuronal membrane (both at the cell body and in the surrounding neurites) and responded strongly to DA application (Fig. 2a-d), with DA affinity similar to what we measured in HEK293T cells. In addition, we obtained the same rank order in terms of the peak response to DA measured in cultured neurons and HEK293T cells. Moreover, the next-generation GRAB<sub>DA</sub> sensors had higher SNR values compared to previous sensors when expressed in neurons (Fig. 2c and 2d and Supplementary Table 1).

Importantly, our new sensors also retained the pharmacological specificity of their respective parent receptors.
 For example, application of the D<sub>1</sub>R-specific and D<sub>2</sub>R-specific antagonists SCH-23390 (SCH) and eticlopride (Etic),

121 respectively, eliminated the corresponding sensors' response to DA (Fig. 2e and Extended Data Fig. 5); interestingly, however, both the  $D_1R$ -specific and  $D_2R$ -specific antagonists inhibited the red fire ant  $D_2R$ -based rDA2m and rDA2h 122 sensors (Fig. 2e and Extended Data Fig. 5b-d), possibly due to low sequence homology between red fire ant D<sub>2</sub>R and 123 124 human  $D_2R$ . Moreover, these new sensors had only a negligible response to a variety of other neurochemicals and 125 transmitters, including glutamate, GABA, levodopa, acetylcholine, serotonin, histamine, octopamine, and tyramine. Importantly, despite the structural similarity between the transmitters DA and norepinephrine (NE) our optimized 126 sensors were approximately 20-80-fold more sensitive to DA than NE (Fig. 2f and Extended Data Fig. 5), indicating 127 128 their extremely high specificity for DA.

Next, we measured the kinetics of our DA sensors using rapid line-scanning confocal microscopy. We locally applied DA and then measured the time constant of the signal rise ( $\tau_{on}$ ) and the time constant of the signal decay following application of the corresponding antagonist ( $\tau_{off}$ ). Our analysis revealed  $\tau_{on}$  values of approximately 80 ms for all DA sensors, and  $\tau_{off}$  values ranging from 0.6-3 s based on differences in each sensor's affinity (Extended Data Fig. 6 and Supplementary Table 1).

We then tested whether our red-shifted cpmApple-based DA sensors are photoactivated by blue light, as shown 134 previously for the cpmApple-based red calcium indicator jRGECO1a<sup>25,32</sup>. Interestingly, unlike most mApple-based 135 136 sensors, we found that bursts of 488-nm blue light had no significant effect on the fluorescence of the rDA1m and rDA2 sensors (Fig. 2g and Extended Data Fig. 4c and 4d and Supplementary Table 1), promising an optimal 137 compatibility with blue-light-activated optogenetic actuators. However, though undesired, RdLight1 and rDA3 138 139 sensors were found to exhibit photoactivation when illuminated with blue light (similar as jRGECO1a), causing a 140 transient increase in fluorescence (Fig. 2g and Extended Data Fig. 4c and 4d and Supplementary Table 1). In addition, 141 the DA-induced increase in fluorescence was stable for up to 2 h in the continuous presence of 100 µM DA when expressed in cultured neurons, with minimal arrestin-mediated internalization or desensitization (Fig. 2h and 142 143 Extended Data Fig. 4e-h). Thus, these sensors are suitable for long-term monitoring of dopaminergic activity.

To examine whether the DA sensors couple to intracellular signaling pathways, we used the luciferase complementation assay<sup>33</sup> and the Tango assay<sup>34</sup> to measure G protein–mediated signaling and β-arrestin signaling, respectively. We found that wild-type receptors showed robust coupling in both assays, whereas all of the DA sensors tested failed to engage either of these GPCR-mediated downstream pathways (Fig. 2i and 2j). We therefore conclude that expressing these receptors likely does not affect cellular physiology.

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Imaging DA dynamics in acute brain slices. Next, we used two-photon imaging to measure the sensitivity of the 150 151 gDA3m and rDA3m sensors for reporting the triggered release of endogenous DA in acute brain slices. We injected 152 the nucleus accumbens (NAc)—which receives dense innervation from midbrain dopaminergic neurons (DANs) with adeno-associated virus (AAV) expressing either gDA3m or rDA3m and then prepared acute brain slices two 153 154 weeks after injection (Fig. 3a and 3b). Electrical stimuli applied at NAc at 20 Hz induced robust transient increases 155 in fluorescence, with the magnitude of the peak response increasing with increasing numbers of stimuli. Moreover, 156 application of the  $D_1R$ -selective antagonist SCH (10  $\mu$ M) eliminated the stimulus-evoked response, confirming that the response is due to DA binding to the sensors. Consistent with our results obtained with cultured cells, we found 157 158 that both the gDA3m and rDA3m sensors had significantly improved sensitivities and responses compared to the 159 corresponding previous-generation sensors (i.e., gDA2m and rDA1m, respectively) (Fig. 3c-e).

Because the fluorescence of the  $D_1R$ -based gDA3m sensor is not affected by  $D_2R$ -specific compounds such as the D<sub>2</sub>R-specific agonist quinpirole or the D<sub>2</sub>R-specific antagonist sulpiride (Fig. 3f), we examined the effect of D2 autoreceptor activity on DA release in slices expressing the D<sub>1</sub>R-based gDA3m sensor. Activation of endogenous D<sub>2</sub>Rs by the D2-specific agonist quinpirole decreased the stimulus-evoked change in gDA3m fluorescence (Fig. 3g and 3h), reflecting presynaptic inhibition via D2 autoreceptors; this decrease was reversed by the addition of sulpiride, and adding the D<sub>1</sub>R-specific antagonist SCH abolished the stimulation-evoked response (Fig. 3g and 3h).

166 To measure DA release in both the cell body and terminals of midbrain DA neurons, we injected AAV expressing gDA3m into the substantia nigra pars compacta (SNc), driving expression in both the SNc cell bodies and dopaminergic 167 terminals; we then prepared acute brain slices (Fig. 3i). We found that low-frequency stimulation (0.33 Hz) elicited 168 169 time-locked transient increases in fluorescence in both the striatum and SNc, while a 40-Hz train of 5 pulses induced 170 a large transient increase in fluorescence, with the signal decay following a slower time course in the SNc compared to the striatum (Fig. 3k). To measure the kinetics of these transients, we performed line-scan microscopy (2 ms/line). 171 Our analysis revealed that the increase in fluorescence upon high-frequency (40 Hz) stimulation had a half-rise time 172 173 (rise  $t_{1/2}$ ) of 20 ± 10 ms and 22 ± 9 ms in the striatum and SNc, respectively; in contrast, the fluorescence signal decayed to baseline significantly slower in the SNc (decay  $t_{1/2}$ ) compared to the striatum, with decay  $\tau_{1/2}$  values of 174 175 209 ± 49 ms and 125 ± 32 ms, respectively (Fig. 3I). This difference in the time course of DA levels may be 176 attributed—at least in part—to differences in dopamine transporter (DAT) expression between the dorsal striatum and midbrain<sup>35</sup>. 177

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179 Validation of our next-generation DA sensors in vivo. Next, we examined whether the increased sensitivity of our 180 DA sensors might be suitable for recording in vivo DA release in the medial prefrontal cortex (mPFC), which receives relatively sparse dopaminergic innervation from the ventral tegmental area (VTA). We therefore virally expressed 181 the optogenetic tool ChrimsonR (ref. <sup>36</sup>) in the VTA and either gDA3h or dLight1.3b in the mPFC; we then optically 182 stimulated the VTA and used fiber photometry to measure the signal in the mPFC (Extended Data Fig. 7). We found 183 that activating VTA neurons elicited robust, transient increases in fluorescence in the gDA3h-expressing mPFC, and 184 185 this increase was blocked by the D<sub>1</sub>R-antagonist SCH (Extended Data Fig. 7b and 7c). In contrast, activating VTA neurons had virtually no effect on dLight1.3b (Extended Data Fig. 7d), indicating that this previous-generation DA 186 187 sensor lacks the sensitivity to detect in vivo DA release in the mPFC. We also found that the gDA3h sensor had discrete, pulse-dependent responses to optogenetic stimulation, and with just one light pulse sufficient to induce a 188 response; in contrast, the less sensitive DA sensor dLight1.3b did not respond in a light pulse number-dependent 189 190 manner (Extended Data Fig. 7e-g).

191 To measure the *in vivo* performance of our new red-shifted GRAB<sub>DA</sub> sensors, we expressed either rDA3m or rDA3mut in the central amygdala (CeA)—a target of dopaminergic projections from the VTA<sup>37,38</sup>—and the light-192 activated channel Channelrhodopsin-2 (ChR2, ref. <sup>39</sup>) in the VTA. We found that activating VTA neurons induced 193 194 robust, transient increases in rDA3m fluorescence in response to 1-s, 5-s, and 10-s light pulses, with the amplitude 195 of the response increasing incrementally with pulse duration. These responses were virtually eliminated by SCH 196 administration and were absent in mice expressing the DA-insensitive rDA3mut sensor (Extended Data Fig. 8b-d). 197 We also expressed either rDA2m or rDA2mut in both the mPFC and the NAc to measure DA release in these regions in response to VTA activation (Extended Data Fig. 8e); for these experiments, the mice were lightly anesthetized to 198 199 reduce the tonic activity of dopaminergic neurons. Under these conditions, activating ChR2-expressing VTA neurons reliably induce pulse number-dependent increases in rDA2m fluorescence in both the densely innervated NAc and 200 201 the sparsely innervated mPFC, with larger signals induced in the NAc compared to the mPFC; moreover, no signal 202 was detected when we expressed the DA-insensitive rDA2mut sensor (Extended Data Fig. 8f-h). Collectively, these 203 results provide compelling evidence that our next-generation DA sensors can be used in vivo to report DA dynamics 204 in several brain regions in real time with high temporal resolution.

To compare the performance of the next-generation DA sensors with the reported GRAB variants, we measured the DA dynamics in the NAc of water-restricted mice when receiving water rewards (Extended Data Fig. 9a and 9b). We found that unpredicted water delivery induced a much larger fluorescence increase of both gDA3m and rDA3m in the NAc compared to gDA2m and rDA1m, respectively (Extended Data Fig. 9c-h). With improved SNR, the gDA3m and rDA3m could readily represent the reward value as the fluorescence response increase with the size of waterdrop accordingly. We next compared the performance of rDA3m and RdLight1 by expressing these sensors in opposite sides of the NAc core, and performed bilateral fiber photometry recording (Extended Data Fig. 9i and 9j).

The rDA3m sensor had a substantially higher fluorescence change than RdLight1 across all water-rewarded sessions
 (Extended Data Fig. 9k and 9l). Taken together, the new DA probes enable DA detection with improved sensitivity
 and precision *in vivo*.

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Simultaneous in vivo imaging of DA and either intracellular cAMP or endocannabinoid signaling during natural 216 behavior. Next, we capitalized on the spectral compatibility between our red-shifted DA sensors and green 217 218 fluorescent sensors in order to monitor multiple signaling events simultaneously in the same location. The NAc plays 219 a key role in reward processing. Therefore, we measured extracellular DA while also measuring intracellular cyclic 220 AMP (cAMP), the downstream messenger activated by DA receptors and a point of convergence for GPCR 221 signaling<sup>40,41</sup>. We expressed both rDA3m and the green fluorescent cAMP indicator G-Flamp1 (ref. <sup>42</sup>) in the NAc of male mice and measured both signals during mating, a naturally rewarding condition<sup>43</sup> (Fig. 4a and 4b). We found 222 that the fluorescence of rDA3m and G-Flamp1 measured in the NAc increased while the male was sniffing the female, 223 224 mounting the female, during intromission, and during ejaculation (Fig. 4c), with a similar half-rise time of  $616 \pm 40$ ms and 698 ± 53 ms, respectively. Interestingly, however, we found that during all four mating stages the rDA3m 225 226 signal preceded the G-Flamp1 signal by approximately 200 ms (Fig. 4d and 4f). Moreover, a session-wide cross-227 correlation analysis revealed that the intracellular cAMP levels measured using G-Flamp1 were closely correlated 228 with the DA signal (Fig. 4e and 4f).

We next examined the extracellular crosstalk between dopaminergic activity and other neurotransmitters. In the 229 230 brain, the basolateral amygdala (BLA) plays an important role in mediating the fear response and processing aversive memories<sup>44</sup>, and previous studies have shown that both DA signaling and the endocannabinoid (eCB) system in the 231 232 BLA participate in anxiety and fear formation<sup>45</sup>. However, the relative timing of DA and eCB signals under stress conditions remains unknown, particularly at high temporal resolution. We therefore expressed both rDA2m and the 233 green fluorescent eCB indicator eCB2.0 (ref. <sup>46</sup>) in the BLA in one hemisphere and measured both signals while 234 applying mild foot shocks to induce stress; as a control, we also expressed and measured rDA1m and eCB2.0 in the 235 other hemisphere (Fig. 4g and 4h). We found that rDA2m and eCB2.0 had reproducible, time-locked transient 236 237 increases in fluorescence upon delivery of a 2-s foot shock; moreover, although the signal produced by eCB2.0 was 238 similar between hemispheres, the signal produced by rDA2m was approximately twice as large as the signal 239 produced by rDA1m (Fig. 4i-I). We also examined the kinetics of the DA and eCB signals and found that although the 240  $\tau_{off}$  rates were similar for DA and eCB (on a order of 4-5 s), the  $\tau_{on}$  rate was significantly faster for DA (~0.8 s) compared to eCB (~2.2 s), with no significant difference between rDA2m and rDA1m (Fig. 4m). This difference 241 between the relatively rapid DA signal and the slower eCB signal is in consistent with the known signaling 242 mechanism of small-molecule transmitters such as DA and lipid neurotransmitters such as eCBs<sup>47,48</sup>. 243

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Simultaneously measuring DA and ACh release in vivo during an auditory Pavlovian conditioning task. 245 Dopaminergic signaling plays a key role in reinforcing learning and memory through the mesocorticolimbic system<sup>49–</sup> 246 <sup>51</sup>. External rewards such as food also elicit characteristic changes in acetylcholine (ACh) levels that promote 247 learning and motivate action<sup>52</sup>. However, the relationship between DA release and ACh release—as well as the 248 249 dynamics of their release in the mesocorticolimbic system during reinforcement learning—are poorly understood. 250 We therefore measured the release of both DA and ACh during auditory Pavlovian conditioning tasks by coexpressing rDA3m and the green fluorescent ACh sensor ACh3.0 (ref. <sup>53</sup>) in both the NAc and the mPFC. Mice were 251 252 head-fixed, water-restricted, and trained to associate a specific auditory cue with either a water reward (associated with tone A) or a punitive mild puff of air applied to the eye (associated with tone B) (Fig. 5a and 5b). Initially, rDA3m 253 mainly responded to the reward, while ACh3.0 responded both to the reward and the punishment, with minimal 254 255 response to the auditory cues (Fig. 5c). After five days of training, however, the mice selectively associated the 256 stimulus-predicted cue (tone A or tone B) with the subsequent delivery of reward or punishment (i.e., water or air 257 puff, respectively). The rDA3m and ACh3.0 signals in the NAc of these trained mice increased in response to the

258 water-predicted tone, but decreased in response to the punishment-related tone, whereas their responses to actual 259 reward or punishment were remained in the current paradigm (Fig. 5c and 5e-f). The development of excitatory responses to the reward cue and inhibitory responses to the punishment cue is consistent with the so-called reward-260 261 prediction-error theory<sup>51</sup>. Interestingly, rDA3m and ACh3.0 signals in the mPFC increased in response to both 262 stimulus-predictive cues and the actual outcomes of both valences in naive mice. After training sessions, unlike what is usually seen in reward prediction error patterns, there was no signal shift (Fig. 5d-f). Furthermore, within 263 brain areas (NAc or mPFC), DA and ACh signals were positively correlated with each other during reward and 264 265 punishment trials (Fig. 5c and 5d), indicating that a similar upstream process regulates DA and ACh release in these two brain regions or a local neuromodulatory effect that one enhances the other<sup>20</sup>. However, these signals were not 266 267 correlated between brain areas (NAc and mPFC; Fig. 5g), suggesting a heterogeneity of neurotransmission in the mesocorticolimbic system. As a control, we found that systemic administration of the D<sub>1</sub>R blocker SCH significantly 268 reduced the rDA3m signal but did not affect the ACh3.0 signal (Extended Data Fig. 10). 269

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Spatially resolved imaging of cortical DA release. Dopaminergic signaling also plays a key role in modulating several 271 272 physiological processes, including motor control and reward perception. The cortex receives dopaminergic 273 innervation from both the SNc and VTA, which send distinct dopaminergic signals<sup>7,54–56</sup>. To test whether our high-274 affinity gDA3h sensor can be used to monitor behavior-related changes in cortical DA levels with high spatiotemporal resolution, we expressed gDA3h in the M1/M2 motor cortex (Fig. 6a) and performed head-fixed in 275 vivo two-photon imaging (Fig. 6b). As DA is thought to be a key regulator of locomotion and aversive events<sup>4,57,58</sup>, 276 277 during imaging, the mouse was placed on a treadmill and gDA3h fluorescence was measured in response to a 70-s 278 bout of forced running (Fig. 6c), an electrical tail shock (Fig. 6d), or an auditory stimulus (Fig. 6e). Interestingly, we 279 observed a robust, rapid, reproducible increase in gDA3h fluorescence aligned to the onset of forced running and tail shock, but not in response to the auditory stimulus (Fig. 6c-g). Similar results were obtained when we expressed 280 gDA3m, whereas dLight1.3b was not sufficiently sensitive to capture these relatively mild changes in DA (Extended 281 282 Data Fig. 11). As a negative control, no response was measured in mice expressing membrane-targeted EGFP (Fig. 6c and 6g; Extended Data Fig. 10). We then examined the spatial patterns of DA release during forced running and 283 284 foot shock on a trial-by-trial basis; interestingly, using select regions of interest (ROIs) we observed distinct patterns 285 during running and shock (Fig. 6c-e). Consistent with this observation, we identified four distinct categories of cell-286 sized ROIs by performing hierarchical cluster analysis to analyze the average response of individual ROIs (Fig. 6h-j). All four categories were observed in all animals tested (Fig. 6k-m). Although most areas had no response, a small 287 subset of responsive regions (representing 0.7% of the entire area) had increases in DA levels during both running 288 289 and shock, while 3.61% and 3.68% of the entire area were associated exclusively with running or shock, respectively 290 (Fig. 6n-o). Taken together, these results show that our gDA3h sensor can be used to map spatially and functionally 291 heterogeneous patterns of DA release in the motor cortex at subsecond resolution.

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#### 293 **DISCUSSION**

Here we used rational design to develop a third-generation series of highly sensitive and highly selective DA sensors suitable for use in *in vivo* multiplex imaging. Our improved red fluorescent DA sensors performed as well as their corresponding green fluorescent counterparts in terms of their sensitivity at detecting DA signals, thereby narrowing the performance gap between red and green fluorescent DA sensors. Moreover, the features of these optimized sensors are compatible with other recently developed optical sensors (e.g., cAMP, eCB, and ACh sensors) for use in monitoring signaling events in the central nervous system in real time.

300 Although several fluorescent DA sensors have been developed using the GPCR-based strategy, the sensors 301 reported to date lack the sensitivity needed to monitor DA release in brain regions with relatively sparse 302 dopaminergic innervation or individual release events, yielding only to the small changes in fluorescence; our highly

303 sensitive series of new DA sensors overcomes this limitation. Moreover, our new series of GRAB<sub>DA</sub> sensors can be 304 used to monitor DA dynamics in vivo in several brain regions such as the NAc, amygdala, and cortex. Importantly, our simultaneous recordings of localized DA release in the NAc and mPFC revealed a lack of synchronized DA release 305 306 from distinct axonal termini. Specifically, in the context of Pavlovian conditioning the well-known theory of prediction-error was observed in the NAc, but not in the mPFC; in the mPFC, the DA signals were consistent with 307 the reported cortical dopaminergic activity in the context of stimulus discrimination<sup>59</sup>. This difference in DA 308 309 dynamics between these two brain regions suggests functional heterogeneity within VTA dopamine neurons, as has been indicated by unique intrinsic properties of cortex-projecting dopamine neurons<sup>60</sup>. In addition, even in a given 310 brain region such as the motor cortex, our two-photon imaging of gDA3h revealed behaviorally related, spatially 311 312 resolved heterogeneity in cortical dopaminergic signaling.

313 Our medium-affinity DA sensors (gDA3m, rDA2m, and rDA3m) are particularly well suited for imaging DA dynamics in brain regions that contain moderate or high DA levels and for monitoring rapidly changing events that 314 315 require a rapid off rate. In contrast, our high-affinity sensors (gDA3h, rDA2h, and rDA3h) can be used to monitor small changes in DA levels, for example in sparsely innervated brain regions. The improved signal-to-noise ratio and 316 317 sensitivity of these sensors have the potential to facilitate the detection of individual release events, thereby greatly 318 enhancing our understanding of the biophysical characteristics of DA release. In addition, the distinct 319 pharmacological profiles of these receptor subtype-based sensors allow for DA imaging while manipulating the 320 activity of specific receptors. These properties are also valuable for studying DA pharmaceutical agents and for screening compounds that target specific DA receptor subtypes. 321

In combination with multicolor fluorescence imaging of other signaling events, our new series of DA sensors can be used to functionally map neurochemical activity. Moreover, this robust set of GRAB<sub>DA</sub> sensors will help pave the way to a deeper understanding of the complexity of the dopaminergic system.

325

#### 326 METHODS

Animals. All procedures for animal surgery and experimentation were performed in accordance and approved by 327 328 the laboratory animal care and use committees of Peking University, the Institutional Animal Care and Use 329 Committee at Oregon Health and Science University, the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Harvard Animal Care and Use Committee. Both male and female postnatal day 0 (PO) 330 Sprague-Dawley rats were used to prepare cultured cortical neurons; P48-P90 wild-type C57BL/6N mice (Beijing 331 Vital River Laboratory), wild-type C57BL/6J mice (Beijing Vital River Laboratory), TH-Cre mice (The Jackson 332 Laboratory; B6. Cg-7630403G23Rik<sup>Tg(Th-cre)1Tmd</sup>/J) and DAT-Cre mice (The Jackson Laboratory; B6.SJL-333 Slc6a3<sup>tm1.1(cre)Bkmn</sup>/J) were used in this study. All animals were housed at 18–23°C in 40–60% humidity under a normal 334 12-h light–dark cycle with food and water available *ad libitum*. 335

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AAV expression. AAV2/9-hSyn-gDA3m (2.8 × 10<sup>13</sup> viral genomes (vg) per ml), AAV2/9-hSyn-gDA3h (8.43 × 10<sup>13</sup> 337 vg/ml), AAV2/9-hSyn-gDA3mut (6.21 × 10<sup>13</sup> vg/ml), AAV2/9-hSyn-gDA2m (5.72 × 10<sup>13</sup> vg/ml), AAV2/9-hSyn-338 dLight1.3b (6.49 × 10<sup>13</sup> vg/ml), AAV2/9-DIO-hSyn-gDA3m (6.2 × 10<sup>13</sup> vg/ml), AAV2/9-EGFP-CAAX(3.5 × 10<sup>13</sup> vg/ml), 339 AAV2/9-hSyn-ACh3.0 (8.0 × 10<sup>13</sup> vg/ml), and AAV2/9-hSyn-eCB2.0 (9.2 × 10<sup>13</sup> vg/ml), AAV2/9-hSyn-GFlamp1 (7.29 × 340 10<sup>13</sup> vg/ml) were packaged at Vigene Biosciences. AAV2/9-hSyn-rDA1m (1.04 × 10<sup>13</sup> vg/ml), AAV2/9-hSyn-rDA2m 341 (6.04 × 10<sup>12</sup> vg/ml), AAV2/9-hSyn-rDA2h (5.31 × 10<sup>12</sup> vg/ml), AAV2/9-hSyn-rDA2mut (5.09 × 10<sup>12</sup> vg/ml), AAV2/9-342 hSyn-rDA3m (3.29 × 10<sup>12</sup> vg/ml), AAV2/9-hSyn-rDA3h (6.36 × 10<sup>12</sup> vg/ml), AAV2/9-hSyn-rDA3mut (6.16 × 10<sup>12</sup> vg/ml), 343 AAV2/9-hSyn-RdLight1 (6.12 × 10<sup>12</sup> vg/ml), AAV2/9-hSyn-hChR2(H134R)-eYFP (5.49 × 10<sup>12</sup> vg/ml), and AAV2/9-hSyn-344 345 ChrimsonR-tdTomato ( $2.52 \times 10^{12}$  vg/ml) were packaged at BrainVTA). Where indicated, the AAVs were either used 346 to infect cultured neurons or were injected in vivo into specific brain regions.

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348 Molecular biology. cDNAs encoding the various DA receptors were cloned from the respective human (hORFeome database 8.1), bovine, sheep, waterbear, bat, cat, monkey, zebrafinch and red fire ant genes (Shanghai Generay 349 350 Biotech). DNA fragments were PCR-amplified using specific primers (Tsingke Biological Technology) with 25-30-bp overlap. Plasmids were generated using Gibson assembly<sup>61</sup>, and all plasmid sequences were verified using Sanger 351 352 sequencing. For screening and characterization in HEK293T cells, the green and red fluorescent DA sensors were 353 cloned into the pDisplay vector (Invitrogen). The IRES-mCherry-CAAX cassette (for expressing green fluorescent 354 sensors) or IRES-EGFP-CAAX cassette (for expressing red fluorescent sensors) was inserted downstream of the sensor gene to serve as a cell membrane marker and to calibrate the sensor's fluorescence. Site-directed 355 356 mutagenesis was performed using primers containing randomized NNB codons (48-51 codons in total, encoding all 357 20 amino acids; Tsingke Biological Technology) or defined codons. For characterization in cultured neurons, the 358 sensor gene was cloned into a pAAV vector under the control of the human synapsin (SYN1) promoter (pAAV-hSyn). For luciferase complementation assay, the receptor-SmBit or sensor-SmBit was generated from  $\beta_2$ AR-SmBit<sup>33</sup>. For 359 the Tango assay, genes encoding the wild-type receptors or the indicated sensors were cloned into the pTango 360 vector<sup>34</sup>. 361

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**Cell culture**. HEK293T cells were cultured at 37°C in humidified air containing 5% CO<sub>2</sub> in DMEM (Biological Industries) supplemented with 10% (vol/vol) FBS (Gibco) and 1% penicillin-streptomycin (Gibco). For experiments, the cells were seeded in 96-well plates or on 12-mm glass coverslips in 24-well plates. At 60-70% confluency, the cells were transfected with a mixture of polyethylenimine (PEI) and plasmid DNA at a 3:1 (w/w) ratio; the culture medium was replaced with fresh medium 6-8 h after transfection, and imaging was performed 24-48 h after transfection. Rat cortical neurons were prepared from PO Sprague-Dawley rats. In brief, cortical neurons were dissociated from the dissected rat cerebral cortex by digestion in 0.25% trypsin-EDTA (Biological Industries) and then plated on poly-D-

lysine-coated (Sigma-Aldrich) 12-mm glass coverslips in 24-well plates. The neurons were cultured in Neurobasal
 medium (Gibco) containing 2% B-27 supplement (Gibco), 1% GlutaMAX (Gibco), and 1% penicillin-streptomycin
 (Gibco) at 37°C in humidified air containing 5% CO<sub>2</sub>. The cultured neurons were transfected with AAVs expressing
 the indicated sensors at 3-5 days *in vitro* (DIV3-5) and imaged at DIV11-14.

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Fluorescence imaging of cultured cells. Before imaging, the culture medium was replaced with Tyrode's solutions 375 containing (in mM): 150 NaCl, 4 KCl, 2MgCl<sub>2</sub>, 10 HEPES, and 10 glucose (pH adjusted to 7.35-7.45 with NaOH). The 376 377 cells then were imaged in a custom-made chamber using an inverted Ti-E A1 confocal microscope (Nikon) and an 378 Opera Phenix high-content screening system (PerkinElmer). The confocal microscope was equipped with a 10x/0.45 number aperture (NA) objective, a 20x/0.75 NA objective, a 40x/1.35 NA oil-immersion objective, a 488-nm laser, 379 and a 561-nm laser. Green fluorescence was collected using a 525/50-nm filter, and red fluorescence was collected 380 using a 595-50nm filter. During imaging, the following compounds were applied via bath application or via a custom-381 382 made perfusion system at the indicated concentrations: DA (Sigma-Aldrich), SCH (Tocris), Etic (Tocris), SKF (Tocris), Quin (Tocris), Glu (Sigma-Aldrich), GABA (Tocris), L-Dopa (Abcam), ACh (Solarbio), 5-HT (Tocris), HA (Tocris), OA 383 384 (Tocris), NE (Tocris), and Sulp (MedChemExpress). To measure the kinetics of the GRAB<sub>DA</sub> sensors, the confocal line-385 scanning mode (2,600 Hz) was used to record the fluorescence response when the cells were locally puffed with DA via a pipette positioned at the cells. Similarly, the decay kinetics were measured by locally puffing cells with the 386 respective antagonist in the presence of saturating DA concentration. The Opera Phenix system was equipped with 387 388 a 20x/1.0 NA, a 40x/0.6 NA objective, a 40x/1.15 NA water-immersion objective, a 488-nm laser, and a 561-nm laser. 389 Green fluorescence was collected using a 525/50-nm emission filter, and red fluorescence was collected using a 390 600/30-nm emission filter, and the fluorescence intensity of the red and green fluorescent sensors was calibrated using EGFP and mCherry, respectively. 391

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393 Spectra and photoactivation measurements. Plasmids expressing the GRAB<sub>DA</sub> sensors were transfected into 394 HEK293T cells in six-well plates (for 1-photon spectra) or on 12-mm coverslips (for 2-photon spectra). For measuring 395 the 1P spectra, the cells were harvested 24-30 h after transfection and transferred to 384-well plates in the absence or presence of 100  $\mu$ M DA. The excitation and emission spectra were then measured at 5-nm increments using a 396 397 Safire2 multi-mode plate reader (Tecan). The fluorescence measured in non-transfected cells was subtracted as background. The 2P spectra of gDA3m were measured at 10-nm increments ranging from 700-1050 nm using an 398 Ultima Investigator 2-photon microscope (Bruker) equipped with a 20x/1.00 NA water-immersion objective 399 400 (Olympus) and an InSight X3 tunable laser (Spectra-Physics). The 2P spectra of rDA3m were measured at 10-nm 401 increments ranging from 820-1300 nm using an A1R MP+ multiphoton microscope (Nikon) equipped with a 25x/1.10 402 NA objective (Nikon) and a Chameleon Discovery tunable laser (Coherent). Laser power was calibrated according to the output power of the tunable 2P laser with various wavelengths. Bursts of 488-nm laser light (1 s duration, 210 403  $\mu$ W, ~0.4 W cm<sup>-2</sup> intensity) were applied to induce blue light–mediated photoactivation. 404

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Luciferase complementation assay. At 50-60% confluency, HEK293T cells were co-transfected with the indicated wild-type receptor or sensor together with the respective LgBit-mG construct. Approximately 24-36 h after transfection, the cells were dissociated using a cell scraper, resuspended in PBS, and transferred to 96-well plates. DA at concentrations ranging from 0.01 nM to 100 μM and 5 μM furimazine (NanoLuc Luciferase Assay, Promega) were then bath-applied to the cells. After a 10-min reaction in the dark at room temperature, luminescence was measured using a VICTOR X5 multi-label plate reader (PerkinElmer).

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413 **Tango assay.** The Tango assay was perform as previously described<sup>34</sup> using HTLA cells (a gift from Bryan L. Roth)

414 seeded in 6-well plates and transfected with plasmids expressing the indicated receptors or sensors. Twenty-four 415 hours after transfection, the cells were collected using trypsin digestion, plated in 96-well plates, and DA was added 416 to the media at concentrations ranging from 0.01 nM to 10 μM. The cells were then cultured for an additional 12 h 417 for luciferase expression; 5 μM Bright-Glo (Fluc Luciferase Assay System, Promega) was then added to the wells, and 418 luminescence was measured using a VICTOR X5 multi-label plate reader (PerkinElmer).

419

420 Two-photon imaging in the NAc in acute mouse brain slices. Adult (6-8 weeks of age) C57BL/6N of both sexes were 421 anesthetized with an intraperitoneal injection of 2,2,2-tribromoethanol (Avertin, 500 mg/kg body weight; Sigma-422 Aldrich), and AAVs were injected (300 nL per injection site at a rate of 40 nl/min) into the NAc using the following 423 coordinates: AP: +1.4 mm relative to Bregma; ML: ±1.2 mm relative to Bregma; and DV: -4.0 mm from the dura. Two weeks after virus injection, the mice were deeply anesthetized, and the heart was perfused with slicing buffer 424 425 containing (in mM): 110 choline chloride, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 7 MgCl<sub>2</sub>, 25 glucose, and 0.5 CaCl<sub>2</sub>. The 426 mice were then decapitated and the brains were immediately removed and placed in cold oxygenated slicing buffer. The brains were sectioned into 300-µm-thick coronal slices using a VT1200 vibratome (Leica), and the slices were 427 428 incubated at 34°C for at least 40 min in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 429 2.5 KCl, 1 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 1.3 MgCl<sub>2</sub>, 25 glucose, and 2 CaCl<sub>2</sub>. Two-photon imaging was performed using either an FV1000MPE 2P microscope (Olympus) equipped with a 25x/1.05 NA water-immersion objective and a mode-430 locked Mai Tai Ti:Sapphire laser (Spectra-Physics) or an Ultima Investigator 2P microscope (Bruker) equipped with a 431 432 20x/1.00 NA objective (Olympus) and an InSight X3 tunable laser (Spectra-Physics). A 920-nm laser was used to 433 excite the gDA3m sensor, and fluorescence was collected using a 495-540-nm filter (for the FV1000MPE microscope) 434 or 490-560-nm filter (for the Ultima Investigator microscope); a 950-nm laser was used to excite the rDA3m sensor, 435 and fluorescence was collected using a 575-630-nm filter (for the FV1000MPE microscope) or a 570-620-nm filter (for the Ultima Investigator microscope). For electrical stimulation, a bipolar electrode (model WE30031.0A3, 436 437 MicroProbes) was positioned near the NAc core under fluorescence guidance, and imaging and stimulation were synchronized using an Arduino board with custom-written software. The stimulation voltage was set at 4-6 V. Where 438 439 indicated, compounds were added by perfusion at a flow rate of 4 ml/min.

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441 Two-photon imaging in the striatum and SNc in acute mouse brain slices. Adult TH-Cre and wild-type mice of both sexes were anesthetized with isoflurane, and the indicated AAVs were injected into the SNc region at the following 442 coordinates: AP: -2.3 mm relative to Bregma; ML: ±1.3 mm relative to Bregma; and DV: -4.5 mm from the dura. 443 444 After 2-3 weeks, the mice were anesthetized with isoflurane, decapitated, and the brain was removed and placed 445 in warm (32-35°C) extracellular solution containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl<sub>2</sub>, 2.4 CaCl<sub>2</sub>, 1.4 NaH<sub>2</sub>PO<sub>4</sub>, 446 25 NaHCO<sub>3</sub>, and 11 dextrose; the solution also contained MK-801 (10  $\mu$ M) to prevent NMDA-mediated excitotoxic 447 damage. Horizontal slices (222-µm thickness) containing the midbrain and striatum were cut using a vibratome in warm extracellular solution and recovered at 30°C for  $\geq$ 30 min before experiments. Two-photon imaging of the 448 449 striatal and SNc slices was performed using a custom-built 2-photon microscope with ScanImage software<sup>62</sup>. Fullframe images (128x128 pixels) were captured at a rate of 4 Hz. Line scans through areas of interest were taken at 450 451 2 ms/line. Images were analyzed using ImageJ (National Institutes of Health) and custom software written using 452 MATLAB.

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In vivo fiber photometry recording in mice. Adult mice were anesthetized with isoflurane, and the indicated AAVs were injected (300 nl total volume) were injected as follows. The NAc was targeted using the following coordinates: AP: +1.4 mm relative to Bregma; ML: ±1.2 mm relative to Bregma; and DV: -4.0 mm from the dura. The mPFC was targeted using the following coordinates: AP: +1.98 mm relative to Bregma; ML: ±0.3 mm relative to Bregma; and DV: -1.8 from the dura. Finally, the BLA was targeted using the following coordinates: AP: -1.4mm relative to Bregma;

- 459 ML: ±3 mm relative to Bregma; and DV: -4.5 mm from the dura. Optical fibers (200 µm diameter, 0.37 NA; Inper) were implanted using the AAV injection site and secured with resin cement (3M). Two weeks after injections, 460 photometry and animal behaviors were recorded using an FPS-410/470/561 photometry system (Inper). In brief, a 461 462 10-Hz (with 20-ms pulse duration) 470/5-nm filtered light-emitting diode (LED) at 20-30 µW was used to excite the 463 green fluorescent sensors, and a 10-Hz (20-ms pulse duration) 561/5-nm filtered LED at 20-30 µW was used to excite the red fluorescent sensors. Alternating excitation wavelengths were delivered, and the fluorescence signals were 464 collected using a CMOS camera during dual-color imaging. To minimize autofluorescence of the optical fiber, the 465 recording fiber was photobleached using a high-power LED before recording. The photometry data were analyzed 466 using a custom-written MATLAB (MATLAB R2022a, MathWorks) program, and background autofluorescence was 467 468 subtracted from the recorded signals.
- 469 *Unpredicted water reward.* Adult female (8-9 weeks in age) C57BL/6J mice were prepared for this experiment. AAV-470 hsyn-rDA3m or AAV-hsyn-RdLight1 virus (300nl for each virus) was bilaterally injected into the NAc. Intraoral cheek 471 fistula implanted and water-restricted mice freely received water delivery (around 10 ul per trial; 25 trials per 472 session; inter-reward-interval = 20 s). To calculate  $\Delta$ F/F<sub>0</sub>, baseline was chosen as the average fluorescence signal 473 during 4.5-5.0 s ahead of water delivery.
- 474 *Mating behaviors.* Adult (8-9 weeks of age) male C57BL/6N mice were used for these experiments. A mixture of 475 AAV-hsyn-rDA3m (300 nl) and AAV-hsyn-Gflamp1 (300 nl) was injected into the NAc as described above. Experienced 476 adult (8-9 weeks in age) ovariectomized (OVX) female C57BL/6N mice were also used to measure the male's mating 477 behaviors. Three days before recording, the OVX female mice received intraperitoneal injections of estrogen (50 µl, 478 0.2 mg/ml on day 1 and 50 µl, 0.1 mg/ml on day 2) or progesterone (50 µl, 1 mg/ml on day 3). The various sexual 479 behaviors were defined as previously described<sup>16</sup>. To calculate  $\Delta F/F_0$ , the baseline was defined as the average 480 fluorescence measured 1-5 min before introducing the female.
- 481 *Foot shock.* Adult (8-9 weeks of age) male C57BL/6N mice were used for these experiments. A mixture of AAV-hsyn-482 eCB2.0 (300 nl) and either AAV-hsyn-rDA2m or AAV-hsyn-rDA1m (300 nl each) was bilaterally injected into the BLA 483 as described above. The mouse was placed in a shock box, and 5 2-s pulses of electricity at an intensity of 0.7 mA 484 were delivered with an interval of 90-120 s between trials. To calculate  $\Delta F/F_0$ , the baseline values was defined as 485 the average fluorescence measured during a 2-s window prior to the first shock trial.
- 486 Pavlovian auditory conditioning task. Adult (8-9 weeks of age) female C57BL/6J mice were used for these experiments. A mixture of AAV-hsyn-gACh3.0 (300 nl) and AAV-hsyn-rDA3m (300 nl) was injected into the NAc and 487 mPFC as described above. A stainless-steel head holder was attached to the skull using resin cement in order to 488 restrain head-fix the animal. For water delivery, an intraoral cheek fistula was implanted in each mouse as previously 489 described<sup>14</sup>. In brief, incisions were made in the cheek and the scalp at the back of the neck. A short, soft silastic 490 491 tube (inner diameter: 0.3 mm, outer diameter: 0.46 mm) connected via an L-shaped stainless-steel tube was then inserted into the cheek incision site. The steel tube was inserted into the scalp incision, and the opposite end was 492 inserted into the oral cavity. The head-fixed mice were habituated to the treadmill apparatus for 2 days (1 h per day) 493 494 before the experiments to minimize potential stress. On the day of the experiment, the Pavlovian auditory conditioning task was performed using two pairs of auditory cues and outcomes, with tone A (2.5k Hz, 70 dB, 2 s 495 496 duration) paired with delivery of 10 µl of 5% sucrose water and tone B (15k Hz, 70 dB, 2 sec duration) paired with 497 deliver of an air puff to the eye. These two pairs were randomly delivered with a 15-20 s randomized inter-trial 498 interval. The water and air puff delivery were precision-controlled using a stepper motor pump and a solenoid valve, 499 respectively. A custom Arduino code was used to control the timing of the pump and solenoid valve and to synchronize the training devices with the photometry recording system. To calculate  $\Delta F/F_0$ , the baseline was defined 500 501 as the average fluorescence signals measured 4.5-5.0 s prior to the first auditory cue.
- 502

503 Fiber photometry recording of optogenetically induced DA release in mice. Adult (8-9 weeks of age) male

C57BL/6N mice were used for these experiments. The mice were anesthetized with isoflurane, and AAV-hsyn-gDA3h
 (300 nl) or AAV-hsyn-dLight1.3b (300 nl) was injected into the mPFC as described above. AAV-hsyn-ChrimsonR tdTomato (300 nl) was also injected into the VTA using the following coordinates: AP: -2.9 mm relative to Bregma;
 ML: ±0.65 mm relative to Bregma; and DV: -4.1 mm from the dura. Optical fibers (200-µm diameter, 0.37 NA; Inper)
 were implanted in the same injection sites and secured with resin cement (3M). Two weeks after virus injection,
 photometry recording was performed using a commercially available photometry system (Thinker Tech, Nanjing,
 China).

511 A 470/25-nm bandpass-filtered (model 65-144; Edmund Optics) LED light (Cree LED) was used to excite the green fluorescent sensors. The emitted fluorescence was bandpass filtered (525/25 nm, model 86-354; Edmund Optics) 512 and collected using a photomultiplier tube (model H10721-210; Hamamatsu). An amplifier (model C7319; 513 514 Hamamatsu) was used to convert the current output from the photomultiplier tube to a voltage signal that was 515 passed through a low-pass filter. The analog voltage signals were then digitized using an acquisition card (National Instruments). To minimize autofluorescence of the optical fiber, the recording fiber was photobleached using a high-516 power LED before recording. The excitation light power at the tip of the optical fiber was 20-30  $\mu$ W and was 517 518 delivered at 100 Hz with 5-ms pulse duration. Background autofluorescence was subtracted from the recorded 519 signals in the subsequent analysis. A 635-nm laser (1-300 mW; LL-Laser, China) was used for optogenetic stimulation 520 with the light power at the tip of the fiber set at 10 mW. Optical stimulation was delivered at 20 Hz (20-ms pulse 521 duration) with a total of 1-20 pulses simultaneously with photometry recording. Where indicated, the mice received an intraperitoneal injection of SCH-23390 (2 mg/kg body weight). 522

When using the red-shifted GRAB<sub>DA</sub> sensors, AAV-hsyn-rDA3m (300 nl) or AAV-hsyn-rDA3mut (300 nl) was 523 524 injected into the CeA using the following coordinates: AP: -1 mm relative to Bregma; ML: ±2.5 mm relative to Bregma; 525 and DV: -4.3 mm from the dura. In addition, AAV-hsyn-rDA2m (300 nl) or AAV-hsyn-rDA2mut (300 nl) was injected in the mPFC and NAc as described above, and AAV-hsyn-ChR2-YFP (300 nl) was injected into the VTA as described 526 above. Two weeks after injection, photometry recording (FPS-410/470/561; Inper) was performed was described 527 528 above. A 488-nm laser (1-160 mW, LL-Laser, China) was used for optogenetic stimulation, with the light power at the tip of the fiber set at 10 mW. Optical stimulation was delivered at 20 Hz (1, 5, or 10 s duration) simultaneously 529 530 with photometry recording. Where indicated, the mice received an intraperitoneal injection of SCH-23390 (6 mg/kg 531 body weight).

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Fiber photometry recording of DA signals in mice receiving water rewards. AAVs carrying GRABDA dopamine 533 sensors were injected into the NAc of DAT-Cre mice (300 nL, unilateral injection; gGRAB<sub>DA2m</sub>: AAV9-hsyn-gDA2m, 534 titer = 2.3x10<sup>13</sup> vg ml<sup>-1</sup>; gGRAB<sub>DA3m</sub>: AAV9-hsyn-gDA3m, titer = 1.3x10<sup>13</sup> vg ml<sup>-1</sup>). For red GRAB<sub>DA</sub> sensors, we injected 535 536 a mixture of AAVs carrying rGRAB<sub>DA</sub> and GFP (3:1 mixture, 300nL total volume; GFP: AAV8-CAG-GFP, titer = 6.7 x 10<sup>12</sup> vg ml<sup>-1</sup>; rGRAB<sub>DA1m</sub>: AAV9-hsyn-rDA1m, titer =  $2.5 \times 10^{13}$  vg ml<sup>-1</sup>; rGRAB<sub>DA3m</sub>: AAV9-hsyn-rDA3m, titer =  $6 \times 10^{12}$  vg ml<sup>-1</sup>). 537 NAc coordinates were, from bregma: AP 1.5 mm, ML 1.7 mm, DV 4.0 mm, angled 4 degrees forward. Mice 1,2,5-10 538 539 also received injections of AAV8-hSyn-FLEX-ChrimsonR-tdTomato (titer = 3.9x10<sup>12</sup> vg ml<sup>-1</sup>) in VTA and SNc (300 nL each, unilateral; coordinates, from bregma: VTA = AP -3.0 mm, ML 0.6 mm, DV 4.3 mm; SNc = AP -3.0 mm, ML 1.6 540 541 mm, DV 4.2 mm). Mice 3,4 received injections of AAV5-CAG-flex-tdTomato (titer = 4.8x10<sup>12</sup>) in VTA and SNc (300 nL 542 each, unilateral). An optic fiber was implanted targeting NAc (400 um diameter, NA = 0.48, Doric lenses).

At least 2 weeks after surgery, mice were water restricted (to 85% of initial body weight) and trained to receive water rewards while head-fixed. After mice consistently licked to water delivery (typically requiring 3 days of training), we recorded dopamine sensor responses to unpredicted delivery of water droplets of various sizes (1, 2, 4, or 8 μL; inter-reward interval = 8-20 seconds, uniformly distributed; 60 trials per session, 15 trials per reward size, randomly interleaved; in some sessions, only 2 or 8 μL of water was given). Photometry signals were collected with a bundle-imaging fiber photometry system (Doric lenses). For green dopamine sensors, we used a blue LED (460-490 nm, 75 μW measured at tip of patch cord) to excite the sensor and the isosbestic wavelength as a control signal

550 (410-420 nm LED, 60 μW). For red dopamine sensors, we used a yellow LED (555-570 nm, 110 μW) to excite the 551 sensor and GFP signals as a control (460-490 nm LED, 75 μW). Imaging was performed at 20 Hz. Photometry data 552 was processed offline as follows.  $\Delta F/F_0 = (F-F_0)/F_0$  was computed by defining  $F_0$  as the 10<sup>th</sup> percentile of each signal 553 within a sliding 30 second window (excluding reward responses, defined as the 5 seconds following reward). Then, 554 linear regression was performed between the sensor signal and the control signal (either isosbestic wavelength or 555 GFP) in ITI periods (5 seconds following reward delivery excluded). The resulting predicted signal was subtracted 556 from the sensor signal to produce the final de-noised signal.

557

558 Two-photon in vivo imaging in mice. Adult (7-8 weeks of age) female C57BL/6N mice were used for these experiments. The mice were anesthetized with isoflurane (3% induction, followed by 1-1.5% maintenance), the skin 559 and skull above the motor cortex were removed, and a metal recording chamber was affixed to the head. AAV 560 expressing either gDA3m, gDA3h, dLight1.3b, or mEGFP (200 nl each, full titer) was then injected into the motor 561 562 cortex using the following coordinates: AP: +1.0 mm relative to Bregma; ML: ±1.5 mm relative to Bregma; and DV: -0.5 mm from the dura). A 4 mm x 4 mm square glass coverslip was then used to cover the opening in the skull. A 563 564 stainless-steel head holder was attached to the skull to head-fix the animal's head and reduce motion-induced 565 artifacts during imaging. Two weeks after virus injection, the mice were habituated for approximately 10 min on the treadmill imaging apparatus to minimize stress. The motor cortex was imaged at a depth of 100-200 µm below the 566 pial surface using Prairie View 5.5.64.100 software with an Ultima Investigator 2-photon microscope (Bruker) 567 equipped with a 16x/0.80 NA water-immersion objective (Olympus) and an InSight X3 tunable laser (Spectra-568 Physics). A 920-nm laser was used for excitation, and a 525/70-nm emission filter was used to collect the 569 570 fluorescence signal at a sampling rate of 1.5 Hz. For the forced running paradigm, running speed was set at 15 cm/s; 571 for the tail shock paradigm, a 3-s electrical shock (0.7 mA) was delivered. For audio stimulation, a 1-s pulse of white noise (80 dB) was delivered. For image analysis, motion-related artifacts were corrected using the EZcalcium motion 572 correction algorithm as described previously<sup>63</sup>. Fluorescence intensity measures at the ROIs was measured using 573 574 ImageJ software. The fluorescent responses were calculated as [(F<sub>raw</sub>-F<sub>baseline</sub>)/F<sub>baseline</sub>], in which F<sub>baseline</sub> was defined as the average fluorescence signal measured for 10 s prior to the behavior onset. The peak response during a 575 576 behavior was calculated as the maximum  $\Delta F/F_0$  measured for 0-5 s after the behavior onset. The brain area was 577 deemed responsive if the average response in a 5-s window surrounding the peak exceeded the sum of the baseline 578 average and the baseline standard deviation. Hierarchical clustering was performed on the average of the fluorescence signals (forced running and shock) for each ROI. Euclidean distance and the Ward linkage metric were 579 used after comparing multiple linkage metrics and clustering algorithms. Variations among individuals were 580 minimized by normalizing the response to the maximum  $\Delta F/F_0$  across ROIs in a given mouse. The hierarchical 581 582 method was used to reduce bias due to predetermining the cluster number.

583

Quantification and statistical analysis. Except where indicated otherwise, all summary data are presented as the 584 585 mean±SEM. Imaging data were processed using ImageJ (1.53c) or MATLAB software (matlab R2020a) and plotted using OriginPro 2020b (OriginLab), GraphPad Prism 8.0.2, or Adobe Illustrator CC. The change in fluorescence ( $\Delta F/F_0$ ) 586 587 was calculated using the formula  $[(F-F_0)/F_0]$ , in which  $F_0$  is the baseline fluorescence signal. The SNR was calculated 588 as the peak response divided by the standard deviation of the baseline fluorescence. Group differences were 589 analyzed using a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test, a one-way ANOVA 590 with Dunnett's multiple comparison test, a two-way ANOVA with Sidak's multiple comparison test, or a two-tailed Student's t-test (GraphPad Prism 8.0.2). Differences were considered significant at p < 0.05; \*p < 0.05, \*\*p < 0.01, 591 592 \*\*\*p<0.001, \*\*\*\*p<0.0001, and n.s., not significant (p>0.05). For all representative images and traces, similar 593 results were obtained for >3 independent experiments.

594

#### 595 **Data availability**

596 The plasmids and sequences used to express the sensors in this study are available from Addgene. Source data will 597 be provided upon reasonable request to the corresponding author.

598

#### 599 Code availability

600 The custom MATLAB codes, Arduino program, and ImageJ programs will be provided upon request to the 601 corresponding author.

602

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612

#### 613 Author contributions

Y.L. supervised the study. Y. Zhuo. and Y.L. designed the study. Y. Zhuo., X.Y., Y.W., G.L., H.W. and Y. Zheng performed 614 the experiments related to the development, optimization, and characterizing of the sensors in cultured HEK293T 615 cells and in neurons. Y. Zhuo., R.C., and T.Q. performed the surgery and two-photon imaging experiments related to 616 the validation of the sensors in acute brain slices. J.T.W. performed the characterization in acute brain slices 617 618 containing the striatum or SNc. H.D., J.W., B. Li., and X.M. performed the *in vivo* fiber photometry recoding during optogenetic stimulation. M.G.C. performed the fiber photometry recording in the mouse NAc for the independent 619 620 validation of in vivo sensor comparison under the supervision of M.W.-U. Y. Zhuo and B. Luo performed the in vivo fiber photometry recording in the NAc during mating behavior. B. Luo performed the in vivo fiber photometry 621 recording during foot shock and the Pavlovian conditioning task with help from H.D. Y. Zhuo performed the in vivo 622 623 two-photon imaging of the motor cortex. All authors contributed to the interpretation and analysis of the data. Y. 624 Zhuo and Y.L. wrote the manuscript with contributions from all authors.

625

#### 626 Competing interests

627 None to declare.

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## Fig. 1 Development and performance of improved dual-color GRAB<sub>DA</sub> sensors

-8

DA (logM)

-6

## 750 Fig. 1 | Development and performance of improved dual-color GRAB<sub>DA</sub> sensors

751 **a**, Schematic illustration showing the principle of next-generation green and red fluorescent dopamine sensors.

b, Schematics of improved dual-color GRAB<sub>DA</sub> sensors. Mutations are indicated with respect to wild-type receptors
 and fluorescent proteins. Igk or HA, N terminus leader sequence.

- c, Spectral profiles of GRAB<sub>DA</sub> sensors. One-photon excitation (top-left), emission (top-right) and two-photon
   excitation (bottom) spectra of indicated sensors in the absence (dashed lines) or presence (continuous lines) DA are
   shown.
- d, Representative images showing sensor expression (top) and fluorescence response to 100 μM DA (bottom) of
   indicated sensor variants. Scale bar, 20 μm.
- 759 e, Titration DA curves of indicated sensors on HEK293T cells. Apparent affinity values (apparent EC<sub>50</sub>) are defined as
- the concentration of half-maximal fluorescence changes (max  $\Delta F/F_0$ ). Data are shown as mean±SEM. *n*=4 wells with 400-500 cells per well for gDA3m, 7 for gDA3h, 10 for gDA2m, 8 for dLight1.3b, 7 for rDA2m, 7 for rDA2h, 6 for
- rDA1m, 12 for rDA3m, 12 for rDA3h, 12 for RdLight1.
- 763 **f**, Group summary of relative brightness of indicated sensors before and after 100 μM DA addition. The brightness
- of green and red sensors was relative to DA2m and rDA1m, respectively. a.u., arbitrary unit. *n*=90 cells from 5
- separate experiments (hereafter denoted as 90/5) for gDA3m, 82/5 for gDA3h, 88/5 for gDA2m, 37/3 for dLight1.3b,
  45/3 for rDA2m, 45/3 for rDA2h, 45/3 for rDA1m, 45/3 for rDA3m, 45/3 for rDA3h, 38/3 for RdLight1. One-way
- 767 ANOVA, post hoc Tukey's test was performed.
- 768 **g**, Group summary of maximal  $\Delta F/F_0$  of indicated sensors in response to 100µM DA. n= 130/7 for gDA3m, 173/8 for
- 769 gDA3h, 200/10 for gDA2m, 80/4 for dLight1.3b, 45/3 for rDA2m, 45/3 for rDA2h, 45/3 for rDA1m, 45/3 for rDA3m,
- 45/3 for rDA3h, 38/3 for RdLight1. One-way ANOVA, post hoc Tukey's test was performed. n.s. p=0.2820 between
   rDA2h and rDA1m.
- 772 **h**, Group summary of signal-to-noise ratio (SNR) of indicated sensors. SNR was calculated based on the response to
- 100μM DA and was relative to DA2m (green) and rDA1m (red), respectively. *n*= 95/5 for gDA3m, 40/3 for gDA3h,
- 96/5 for DA2m, 31/3 for dLight1.3b, 45/3 for rDA2m, 45/3 for rDA2h, 45/3 for rDA1m, 45/3 for rDA3m, 45/3 for
- rDA3h, 38/3 for RdLight1. One-way ANOVA, post hoc Tukey's test was performed. p=0.0016 between rDA2h and
- 776 rDA1m.
- 777

# Fig. 2 Characterization of new GRAB<sub>DA</sub> sensors in cultured cells



### 778 Fig. 2 | Characterization of new GRAB<sub>DA</sub> sensors in cultured cells

a, Representative images of gDA3m or rDA3m expressing-primary cultured rat cortical neurons showing cell
 membrane localization. Scale bar, 100 μm (left) and 20 μm (right).

b, Representative images showing expression and fluorescence response of gDA3m and of rDA3m in dissociated
 cortical neurons. Scale bar, 50 μm. Similar results were observed more than 30 cells.

- c, Titration DA curves (left) and group summary of peak response (top-right) and relative SNR (bottom-right) of
   indicated green dopamine sensors on cultured neurons. Left, n= 60, 60, 120, 60 neurons for gDA2m, gDA3m, gDA3h
   and dLight1.3b. Right, n=130, 175, 200, 80 neurons respectively. One-way ANOVA, post hoc Tukey's test was
   performed.
- 787 **d**, Titration DA curves (left) and group summary of peak response (top-right) and relative SNR (bottom-right) of
- indicated red dopamine sensors on cultured neurons. Left, *n*=30 neurons for all sensors. Right, *n*=60 neurons for all
   sensor variants. One-way ANOVA, post hoc Tukey's test was performed. For SNR, n.s. p=0.7632 between rDA2h and
   rDA1m. \*\*\*p=0.005 between rDA2m and rDA1m.
- 791 e, Pharmacological specificity of gDA3m, gDA3h, rDA2m and rDA3m in neurons. SCH-23390 (SCH), D<sub>1</sub>R antagonist;
- eticlopride (Etic), D<sub>2</sub>R antagonist; SKF-81297 (SKF), D<sub>1</sub>R agonist; quinpirole (Quin), D<sub>2</sub>R agonist; glutamate (Glu);
   gamma-aminobutyric acid (GABA); levodopa (L-Dopa); acetylcholine (ACh); serotonin (5-HT); histamine (HA);
- octopamine (OA); tyramine (TA). Antagonists were applied at 10µM, others at 1µM. *n*=4 wells for gDA3m, *n*=5 wells
  for gDA3h, *n*=3 wells for rDA2m and rDA3m. Each well contains 100-200 neurons. One-way Anova, post hoc
  Dunnett's test was performed. gDA3m, n.s. p=0.0987 between DA and DA+Etic. gDA3h, n.s. p=0.2032 between DA
- and DA+Etic. rDA3m, n.s. p=0.8251, 0.9993 between DA and DA+Etic, or SKF.
- f, Titration curves of indicated sensors for the response to DA or NE in cultured neurons. Data are shown as
   mean±SEM. *n*=60 neurons from 3 experiments.
- 800 **g**, Representative traces (left) and group summary of  $\Delta F/F_0$  (right) of indicated sensors upon blue-light illumination.
- 801 *n*=70, 13, 17, 8, 8 cells for jRGECO1a, rDA1m, rDA2m, RdLight1 and rDA3m, respectively. One-way ANOVA, post hoc
- Tukey's test was performed. n.s. p=0.7180 between jRGECO1a and rDA3m, p=0.9927 between rDA3m and RdLight1,

803 \*\*p=0.0083 between rDA2m and rDA3m.

- h, Representative images (left) and quantification (right) of the change in sensor fluorescence in response to 2-h
   application of 100μM DA. Scale bar, 20 μm. n=3, 9 cultures for gDA3m and rDA3m. One-way ANOVA test was
   performed for DA-containing groups. n.s. p=0.4375, 0.1895 for gDA3m, rDA3m.
- i, G-protein coupling was measured using luciferase complementation assay in cells expressing wild-type receptor,
   sensor or no receptor (G<sub>s/i</sub>-LgBit alone, ctrl). Data are shown as mean±SEM. *n*=3 cultures.
- j, β-arrestin coupling was measured with Tango assay in cells expressing receptor or sensor. Data are shown as
   mean±SEM. *n*=3 cultures. N.A., not applicable.





## Fig. 3 | GRAB<sub>DA</sub> sensors enable endogenous DA detection in mouse acute brain slices

- 812 **a**, Schematic illustration depicting the experimental design for panel **b-e**.
- **b**, Representative fluorescence images showing gDA3m or rDA3m expression in the NAc region. Scale bar, 100 μm.
- 814 **c**, Example fluorescence response to indicated electrical stimulation measured in the sensor-expressing brain slices.
- 815  $\,$  The dashed circles indicate the ROI used to analyze the responses. Scale bar, 100  $\mu m.$
- 816 **d-e**, Representative traces (left) and group summary (right) of the change in sensor fluorescence in response to 817 electrical stimulation (1 ms per pulse). *n*=8 slices from 5 mice (8/5) for gDA3m and *n*=5/4 for rDA3m. The insets 818 show quantification of the  $\Delta$ F/F<sub>0</sub> of indicated sensors in response to 1 pulse stimulation. Data replotted from 819 previous results of DA2m and rDA1m<sup>16</sup>. Two-tailed Student's t-test was performed. p=0.0429 and 0.0138 for gDA3m 820 and rDA3m, p=0.0076 between gDA3m and gDA2m, p=0.0008 between rDA3m and rDA1m.
- f, Normalized fluorescence change in gDA3m to the indicated compounds (each at 1µM). Sulpiride (Sulp), D<sub>2</sub>R
   antagonist. n=3 wells with 500-600 cells per well. One-way Anova, post hoc Dunnett's test was performed. p=0.9813,
   0.8848 between DA and DA+Quin, or DA+Sulp.
- g. Representative pseudocolored images (top) and traces (bottom) of the fluorescence response of gDA3m to
   electrical stimuli (5V, 3ms) in ASCF and following drug treatments (each at 1µM). The yellow dashed line indicates
   the electrode placement. The dashed circles indicate the ROI used to analyze the responses. Scale bar, 100 µm.
- 827 **h**, Group summary of fluorescence response of gDA3m to electrical stimulation in either ACSF or the indicated drugs.
- 828 *n*= 6 slices from 5 mice. One-way ANOVA, post hoc Tukey's test was performed. p=0.00233, p>0.99, p<0.0001
- 829 between ACSF and Quin, Sulp or SCH; p=0.00231 between Quin and Sulp.
- 830 i, Schematic illustration depicting the experiment design of panel j-l.
- **j**, Representative fluorescence images showing gDA3m expression in the striatum (top) and SNc (bottom) in control
- condition (left) and when delivering 5-pulse stimulation at 40 Hz (right). Scale bar, 5 μm. The yellow lines indicate
   the hotspot for line-scanning.
- 834 **k**, Full-frame fluorescence response in response to a series of indicated electrical stimulation (0.5 ms per pulse). The
- signals were relative to the peak of the first stimulation. Data are shown as mean±SEM. n = 6 slices for striatum, n
  = 9 slices for SNc.
- 837 **I**, Representative line-scan (500 Hz) image in the striatum (top) and the SNc (bottom) and averaged normalized 838 fluorescence traces in response to multi-pulses 40 Hz stimulus. Data are shown as mean $\pm$ SEM. *n* = 6 areas for 839 striatum and SNc, respectively.
- 840

## Fig. 4 | Multiplexed measurements of DA and other neurochemical signals during natural behaviors



- **Fig. 4 | Multiplexed measurements of DA and other neurochemical signals during natural behaviors**
- 842 **a**, Schematic illustration depicting the experimental design for panel **b-f**.
- b, Histological verification of rDA3m and GFlamp1 expression in NAc. DAPI, 4,6-diamidino-2-phenylindole. Scale bar,
   1 mm.
- c, Example traces (top) and zoom-in traces of rDA3m (red) and GFlamp1 (green) signals ( $\Delta F/F_0$ ) simultaneously measured during the indicated stages of mating.
- d, Group-averaged rDA3m and GFlamp1 fluorescence aligned to event onset for all mice. The signals were
   normalized to respective maxima and minimum. *n*=4 mice.
- e, Cross-correlation between simultaneously recorded rDA3m and GFlamp1 signals during indicated stages and of
   shuffle group. n = 4 mice.
- 851 **f**, Group summary of peak correlation coefficient (top) and time lag of cross-correlation peak (bottom) between
- rDA3m and GFlamp-1 signals across mating stages. The black lines indicate mean±SEM. *n* = 4 mice. One-way ANOVA,
   post hoc Tukey's test was performed.
- 854 g, Schematic illustration depicting the experimental design for panel g-k.
- h, Histological verification of rDA2m and eCB2.0 expression (left side), and rDA1m and eCB2.0 expression (right side)
   in BLA. Scale bar, 1mm.
- i, Pseudocolored fluorescence responses of rDA2m and eCB2.0 simultaneously measured in the BLA to ten
   consecutive 2-s foot shock at 0.7 mA.
- **j**, Average traces of the change in rDA2m (top) and eCB2.0 (bottom) fluorescence from a mouse. The grey shaded area indicates the application of electrical foot shock. Data are shown as mean±SD.
- 861 **k**, Same as (**h**) with simultaneously recorded contralateral rDA1m and eCB2.0 signals.
- I, Group summary of the peak change in fluorescence of indicated sensors to 2-s foot shock. Peak responses were
   calculated as the maxima during 0-5 s after foot shock initiation. *n*=4 mice. Paired two-tailed Student's t-test was
   performed. \*p=0.0390 between rDA2m and rDA1m; p=0.7019 between eCB2.0 groups.
- m, Summary of rise and decay time constants measured for the fluorescence change of indicated sensors in
   response to foot shock. The inset shows the example average trace of rDA2m and eCB2.0 signals that were
   normalized to respective maxima and minimum. *n*=4 mice. One-way ANOVA, post hoc Dunnett's test was performed.
- 868 Rise time, p=0.5742 and 0.0087 between rDA2m and rDA1m, or eCB2.0. Decay time, p=0.2180 for all groups.
- 869

## Fig. 5 | DA and ACh signals in mouse NAc and mPFC during an auditory Pavlovian conditioning task



## Fig. 5 | DA and ACh signals in mouse NAc and mPFC during an auditory Pavlovian conditioning task

a, Schematic illustration depicting experimental design for panel **b-g**.

b, Example traces of rDA3m (red) and ACh3.0 (green) signals (ΔF/F<sub>0</sub>) simultaneously measured in the NAc (top) and

873 mPFC (bottom) from a trained mouse during four consecutive trials. The audio, water and puff delivery are indicated 874 above.

- 875 **c**, Representative time-aligned pseudocolored images and averaged traces of rDA3m and ACh3.0 fluorescence from
- a mouse in naïve (top) and trained (bottom) state (c1). Shown are 100 consecutive trials (mean±SD) in one mouse.
- 877 The grey shaded area indicates the application of audio. The dashed line indicates the delivery of water or puff.
- Session-wide correlation between rDA3m and ACh3.0 signals across naïve (top) and trained sessions (bottom) (c2).
   *n*=3 mice.
- 880 **d**, same as (c) with simultaneously recorded rDA3m and ACh3.0 signals in the mPFC.
- e, Group-averaged rDA3m (top) and ACh3.0 (bottom) fluorescence in the NAc (left) and mPFC (right) for all mice
   under naïve and trained state. Water or puff sessions are indicated above. n=3 mice.
- **f**, Group analysis of the normalized average change of rDA3m (top) and ACh3.0 (bottom) signals to US (left) and CS (right) in different sessions. The average response was calculated as the average  $\Delta F/F_0$  in the 1 s after the behavior
- onset. The grey points indicate data from individual animal; Average and SEM are shown by data points with state-
- 886 represented color. *n*=3 mice. Two-way ANOVA, post hoc Sidak's test was performed between water and puff sessions
- and between naïve and trained state. Responses in the trained mice NAc, p=0.0145 (rDA3m-US), 0.0287 (rDA3m-
- CS), 0.0371 (ACh3.0-US), 0.0356 (ACh3.0-CS) between water and puff trial; rDA3m water trial CS response in the
- 889 NAc, p=0.0290 between naïve and trained.
- g, Session-wide cross-correlation between rDA3m (top) or ACh (bottom) signals recorded in the NAc and mPFC. n=3
   mice.
- 892

## Fig. 6 Spatially resolved heterogeneous cortical DA dynamics in mice



#### 893 Fig. 6 | Spatially resolved heterogeneous cortical DA dynamics in mice

- a-b, Schematic illustration depicting the strategy for virus injection and head-fix two-photon imaging in the mouse
   motor cortex. Example fluorescence image showing gDA3h expression in the M1/M2 region in a coronal brain slice
   (a). Scale bar, 500 μm. Representative in vivo 2P image of the layer 2/3 in the M1/M2 cortex showing gDA3h
   fluorescence (b). Scale bar, 100 μm.
- c, Schematic cartoon illustrating the forced running experiments (c1), representative pseudocolored response
   images (c2) and traces measured at indicated ROIs during three consecutive trials (c3) in the head-fix mice
   expressing gDA3h (top) or membrane-targeted EGFP (mEGFP, bottom). The white squares indicate ROI to analyze
   signals. Scale bar, 50 μm.
- d-e, Similar to (c) except mice were subjected to 3-s tail shock, 0.7mA (d) or 1-s audio stimulation (e). Two-photon
   imaging was performed in the same region across different behaviors.
- f, Group summary of the peak fluorescence change measured in the motor cortex in mice expressing gDA3h or
   mEGFP in response to indicated stimulus. *n*=5 mice for gDA3h and 4 mice for mEGFP. Two-tailed Student's t-test was
   performed. p<0.0001 between gDA3h and mEGFP for running and p=0.0017 for shock.</li>
- g, Group summary of the rise and decay time constant of the gDA3h signals in response to forced running and tail
   shock. *n*= 5 mice.
- 909 **h**, Example image showing the spatial responding pattern to forced running, tail shock, merge and ROI selection.
- 910 The responding area was defined according to fluorescence signals during indicated behaviors. ROIs (30 μm x 30
- 911 μm) were randomly selected inside and outside of responding area and colored according to the type of 912 fluorescence responses.
- 913 **i**, Hierarchical clustering of ROI-specific responses to running and shock from a mouse. ROIs were indicated in (**h**).
- 914 **j**, Same to (**h**) with data collected from another two mice.
- 915 **k**, Population data showing hierarchical clustering of ROI-specific response. *n*=91 ROIs from 5 mice.
- I-m, Average (bold lines) and individual (thin lines) traces (I) and quantifications of response amplitudes (m) from
   ROIs within each cluster in (k) during different behaviors. The black lines indicate the application of indicated
   stimulus. n=26, 24, 21 and 20 ROIs from 5 mice for each cluster. One-way ANOVA, post hoc Tukey's test was
   performed. Forced running, p=0.0128 between *none* and *shock*; p=0.0585 between *running* and *both*. Tail shock,
- 920 p=0.9989 between *none* and *running*; p=0.6474 between *shock* and *both*. Audio, n.s. p=0.0766 for all clusters.
- 921 **n**, Percentage of area that was responsive to the indicated stimulus as in (**h**).
- 922 **o**, Venn diagram of the imaged motor cortex area that was responsive to the indicated stimulus. Data collected from
  923 5 mice.
- 924

### Extended Data Fig. 1 | Strategy for optimizing and screening the green GRAB<sub>DA</sub> sensors.



561 VPFCGSGETKPFCIDSITFDVFVWFGWANSSLNPIIYAFNCNVVLIPHAVGSSEGLKKEEAVGIAKPLEKLSPALSVIL 640

cpEGFP

641 DYDTDVSLEKIQPITQNGQHPT \*

IgK GPCR

NE-loop linker

mutated sites

#### 925 Extended Data Fig. 1 | Strategy for optimizing and screening the green GRAB<sub>DA</sub> sensors.

- **a**, A flowchart showing the development process (top) and screening (bottom) of the gDA3m sensor.  $\Delta F/F_0$ represents the fluorescence change of sensor variants in response to 100  $\mu$ M DA. The ICL3 domain of human D<sub>1</sub>R was replaced by the entire ICL3 (including linker and cpGFP) derived from several existing GPCR-based sensors (GRAB<sub>DA2m</sub>, GRAB<sub>NE</sub> and dLight1.3b). Newly generated candidate with highest  $\Delta F/F_0$  after ICL3 replacement (ICL3 from dLight1.3b) was then selected for further cpEGFP optimization, linker optimization and GPCR engineering.
- 931**b**, A flowchart showing the development process and screening of the gDA3h sensor. The ICL3 domains of dopamine932D1 receptors from diverse species were replaced by the entire ICL3 from  $GRAB_{NE}$  sensor. Further optimization on933the best chimera candidate (with bovine  $D_1R$  backbone) includes ICL3 truncation for an optimal length, cpGFP934the best chimera candidate (with bovine  $D_1R$  backbone) includes ICL3 truncation for an optimal length, cpGFP
- 934 mutation for improved brightness and response, as well as GPCR engineering for affinity tunning.
- 935 **c**, Amino acids sequence of the gDA3m sensor. The mutations adopted in the gDA3m sensor are indicated by the 936 black box. The serine residue at position  $229^{5.42}$  in the human D<sub>1</sub>R was mutated to an alanine to generate the gDA3-937 mut sensor (indicated by the gray box).
- 938 **d**, Amino acids sequence of the gDA3h sensor. The mutations adopted in the gDA3h sensor are indicated by the
  939 black box.
- 940

# Extended Data Fig. 2 | Strategy for optimizing and screening the red GRAB<sub>DA</sub> sensors.



## С

# rGRAB<sub>DA2m</sub>

1	METO	DTLL	LWV	LLLW	VPG	STGD	TSL	ΥΚΚΥ	GTTG	INRL	NYS	VLVNI	ΤΑΥ	DGG	AGLN	ILSS	VNC	r s s i	VAGG	AVR	PGEC	AGTA	80
81	GVD	EKSN	IANS	WWAL	ILV	IVPC	LTL	FGNV	LVIL	AVVK	ERT	LQTV	NYFI	VSL	AVAC	LLV	AVLN	/MPF	Ανγν		GSWS	LPGF	160
161	VCDI	YIA	MDV	TCST	SSI	FNLV	AIS	IDRY	ΙΑΥΤ	QPIK	YAK	HKNNF	RVWL	TIL	LVWA	ISA.	AIGS	SPIV	LGLN		DRIP	DACL	240
241	FYNT	(rDA FDFI	2mut: IYS	C173 <sup>3.36</sup> S L S S	A, S25 FYI	0 <sup>5.42</sup> N) PCII	MVF	LYYN	IFKA	LRNF	R A KL	FRAS	VCSE	VMY	PEDG	BALK	SEIV	VKGL	RLKC	GGH	YAAE	<b>үктт</b>	320
321	YKAI	KPV	QLP	GAYI	VDI	KLDI	VSH	NEDY	TIVE	QCEF	RAEA	RHSTO	GMDE	LYK	GGTG	GSL	vsko	BEED	NMAI	IKE	FMRF	кунм	400
401	EGS	/NGH	IEFE	IEGE	GEG	RPYE	AFQ	TAKL	кутк	GGPL	PFT	NDILS	PQFN	IYGS	ΚΑΥΙ	KHP.	ADIF	PDYF	KLSF	PEG	FRWE	RVMN	480
481	FEDO	GII	HVN	QDSS	LQD	GYFI	YKV	KLRG	TNFP	PDGF	VMQ	кктма	WELI		(rDA2h RKAT	: T535 <sup>6</sup> KPL	<sup>.34</sup> S) A I V L	GVF	LICV	VPF	FTCN	IMDA	560
561	ІСТИ	(LTK	ACQ	PGVT	AFI	vтsw	LGYI	MNSF	VNPV	ΙΥΤΛ	/FNP	EFRKA	FRKL	.   S	*								640
	lg	ĸ	(	GPCR		linke	er	cpr	mApple	Г.	muta	ted site:	3										

#### d

# rGRAB<sub>DA3m</sub>

1	MK	ТΙ	I A	LS`	Y I <u>I</u>	FCL	VF/	A D Y	<u> </u>	DD	DAI	MR1	ΓLΝ	ΤS	AMD	GTO	3 L V	VEI	RDF	SVF	R I L	TA	CFL	SLI		STI	LLG	NT	LVC	AA	VIR	FRF	ILRS	80
						(rDA	3h: \	<b>/97</b> 2.	<sup>.53</sup> l)																									
81	KV	ΤN	FF	VI	SLZ	AVS	DLI		V L	VМ	PW	KA۱	/ A E	IA	GFW	PFC	3 S F	CN	ıwv	AFC	ΝΙС	CS.	TAS	ILI	NLC	VI:	SVD	R Y	NA I	SS	PER	YEF	кмт	160
					-	-		-							-							-	-	(rD	A3mı	it: S	2225.4	<sup>12</sup> A. S	S226⁵	.46P)	1			
161	PK	ΔΔ	ΕI	119	s v /	ΔWT	I SV		SE	IP	vo	I SV	инк	ΔΚΙ	ртя	PSI	GN	ΔΤ	SI A	E T I	ח ח	CDS	SSI	SR	ΓΥΔ	LS	s s v	IS	FYI	ΡV		u vla	ТУТБ	240
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241	IV	РI	<u>۸</u>	ĸkl		S E M	vsi			ED	GA		2 5 1	KK		IKI	າດດ	ну	^ ^ E	VKI	гтч	KAI	ĸĸĎ	VO	PC	۸V	ם ע ו	IK	וחו	VS		I V D		320
241		K I .	~ ~		- 0 .		v 01		A I F		U A			N N V	GER		000			VI		NA1	NNF	V Q I	0	~ '		IN		v 0				. 520
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321	QU	ER	AE	GRI	13	166	וטואו		r n G	GI	GG	5 L \	/ 5 N	GEI	EDN	IN A	IIN	EFI	WRF	N V F	1 IVI E	63	VNG	HEI		EGI	EGE	GR	P T E	AF	QIA	<b>NL</b>	VIN	400
401	GG	ΡL	ΡF	AWI	DII	SP	QFN	M Y G	3 S K	AY	IK	HPA	AD I	P D	YFK	S S F	F P E	GFI	RWE	RVN	/NF	EDO	GGI	IH	/NQ	DS	SLQ	DG	VFI	YK	VKL	. R G 1	'NFP	480
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481	PD	GP	VМ	QKI	КΤΙ	MGW	EH	RKE	DL	KR	ETI	K V L	_ К Т	LS	V I М	GVF	= V C	CW	LPF	FIL	NC	ILI	PFC	GSO	GΕΤ	QP	FCI	DSI	NTF	DV	FVW	/F GV	VANS	560
561	SL	NΡ	I I	YAI	FN/	A D F	RK	A F S	STL	LG	CYI	RLC	СРА	TNI	ΝΑΙ	EΤ\	/si	NNI	NGA	AMF	ss	HHI	EPR	GS	ISK	ECI	NLV	ΥL	IPH	AV	GSS	EDL	. K K E	640
641	EA	AG	I A	RPI	LE	< L S	ΡΑΙ	LSV	/   L	DY	DTI	DVS	SLE	KIG	QPI	TQN	١GQ	HP	Т *															720
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		HA			FL	AG		G	PCR	2		lin	ker		С	pmA	pple		Πm	nutate	ed si	ites												
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### 941 Extended Data Fig. 2 | Strategy for optimizing and screening the green GRAB<sub>DA</sub> sensors.

a, A flowchart showing the development process and screening of the rDA2m sensor. The ICL3 domains of dopamine
 D2 receptors from diverse species were replaced by the entire ICL3 from rGRAB<sub>DA</sub> sensor. Further optimization on

the best chimera candidate (with ant  $D_2R$  backbone) includes interface mutation (loop adjacent sites on the TM5

and TM6 of GPCR), receptor N-terminus truncation and GPCR mutation.

946 **b**, A flowchart showing the development process and screening of the rDA3m and rDA3h sensor. The cpRFP module,

947 RFP-GPCR interface, linker region and GPCR backbone of previously reported red dopamine sensor RdLight1 were
 948 systematically optimized.

949c, Amino acids sequence of the rDA2m sensor. The mutations adopted in the rDA2m sensor are indicated by the950black box. The tyrosine residue at position  $535^{6.34}$  in the ant D2R was mutated to serine to generate the high affinity951rDA2h sensor (indicated by the magenta box). The cysteine to alanine mutation at position  $173^{3.36}$  and serine to952asparagine at position  $250^{5.42}$  were adopted to generate the rDA2-mut sensor (indicated by the gray box).

- 953 **d**, Amino acids sequence of the rDA3m sensor. The mutations adopted in the rDA3m are indicated by the black box.
- 954 The valine residue at position  $97^{2.53}$  in the human  $D_1R$  was mutated to isoleucine to generate the high affinity rDA3h
- 955 sensor (indicated by the dark red box). The serine to alanine mutation at position 222<sup>5.42</sup> and serine to proline at

956 position 226<sup>5.46</sup> were adopted to generate the rDA3-mut sensor (indicated by the gray box).

957

## Extended Data Fig. 3 | Perfomance of DA-insensitive mutant sensors.



#### 958 Extended Data Fig. 3 | Performance of DA-insensitive mutant sensors.

- a, Representative images showing sensor expression (top) in HEK293T cells and fluorescence response to 100 μM
   DA (bottom) of indicated sensor variants. Scale bar, 20 μm.
- 961 **b**, Group summary of maximal  $\Delta F/F_0$  in response to 100µM DA (left) and titration DA curves (right) of indicated
- 962 sensors in HEK293T cells. Left, n=6, 6, 15, 15, 12, 3 wells for gDA3m, gDA3mut, rDA2m, rDA2mut, rDA3m and
- 963 rDA3mut. Each well contains 400-500 cells. Two-tailed Student's t-test was performed. Right, *n*=3 wells (with 400-
- 964 **500** cells per well) for each group.
- 965 **c**, Representative images showing sensor expression (top) in cultured neurons and fluorescence response to 100
- $966~\mu M$  DA (bottom) of indicated sensor variants. Scale bar, 50  $\mu m.$
- 967 **d**, Group summary of maximal  $\Delta F/F_0$  of indicated sensors in response to 100µM DA in cultured neurons. *n*=60
- 968 neurons from 4 cultures for rDA2mut, n=30/2 for others. Two-tailed Student's t-test was performed.
- 969

# Extended Data Fig. 4 | Characterization of GRAB<sub>DA</sub> sensors in HEK393T cells and cultured neurons.



#### 970 Extended Data Fig. 4 | Characterization of GRAB<sub>DA</sub> sensors in HEK393T cells and cultured neurons.

- 971 **a**, Excitation and emission spectra for the indicated sensors in the absence and presence of DA.
- 972 **b**, The effect of pH on GRAB<sub>DA</sub> signals. Left, quantification for DA- or buffer-induced fluorescence responses of
- 973 indicated sensors under different extracellular pH conditions. n=3 wells with 400-500 cells per well. Right,
- 974 quantification for the relative buffer-induced fluorescence response of indicated sensors. The sensor-expressing
- 975 HEK293T cells were gently permeabilized by detergent Triton-X100 (0.3% for ~ 5 minutes). The fluorescence
- 976 intensity of pH 6.95 was set as  $F_0$  and the relative fluorescence changes in each pH value were plotted. n=3 wells 977 (with ~2 x 10<sup>5</sup> cells per well).
- 978 **c-d**, Representative traces (**c**) and group summary of  $\Delta F/F_0$  (**d**) of indicated sensors upon blue-light illumination. n=
- 979 18, 14, 18, 16 cells for rDA2h, rDA2mut, rDA3h and rDA3mut; other data replotted from Fig. 2g.
- e-h, Representative images (left) and quantification (right) of the change in sensor fluorescence in response to 2-h
   application of 100μM DA. Scale bar, 20 μm. *n*=3, 8, 5, 4 cultures for gDA3h, rDA2h, rDA2m and rDA3h. One-way
   ANOVA test was performed for DA-application groups. n.s. p=0.5104, 0.2183, 0.4101, 0.0652 for gDA3h, rDA2h,
   rDA2m and rDA3h.
- 984





## 985 Extended Data Fig. 5 | Pharmacological profiles of new GRAB<sub>DA</sub> sensors measured in cultured cells.

986 a, Titration curves of indicated sensors for the response to DA or NE in HEK293T cells. *n*=3 cells with 400-500 cells
 987 per well.

988 **b**, The normalized  $\Delta F/F_0$  in sensor-expressing HEK293T cells in response to the indicated compounds. Antagonists 989 were applied at 10µM, others at 1µM. *n*=4 wells for gDA3m and gDA3h, *n*=3 wells for others.

990 **c**, The normalized  $\Delta F/F_0$  in rDA2m-expressing HEK293T cells in response to indicated DA agonists. Bromocriptine 991 (Bro), Rotigotine (RTG), D<sub>2</sub>R/D<sub>1</sub>R agonists; Ropinirole (RPR), Quin, D<sub>2</sub>R-specific agonists; Fenodopam (FD), SKF, D<sub>1</sub>R-

specific agonist. All chemicals were bath-applied in 100 µM. One-way Anova, post hoc Dunnett's test was performed.
n.s. p=0.1074 between DA and Bro.

994 **d**, Titration curves of indicated dopamine receptor antagonists. The fluorescence intensity in the presence with 10

995  $\mu$ M DA was set as F<sub>0</sub> and the relative fluorescence changes under indicated compound concentration were plotted.

996 **e-f**, Pharmacological specificity (left) and titration curves of indicated sensors for the response to DA or NE (right) 997 in cultured neurons. Left, antagonists were applied at  $10\mu$ M, others at  $1\mu$ M. *n*=3 wells. One-way Anova, post hoc

998 Dunnett's test was performed. rDA2h, n.s. p=0.9998, 0.1458 between DA and DA+SCH, or DA+Etic; rDA3m, n.s.

999 p=0.9591, 0.1309 between DA and DA+Etic, or SKF.

1000

# Extended Data Fig. 6 | Kinetics measurement of new GRAB<sub>DA</sub> sensors in HEK293T cells.



#### 1001 Extended Data Fig. 6 | Kinetics measurement of new GRAB<sub>DA</sub> sensors in HEK293T cells.

1002 **a**, Schematic illustration showing the local perfusion system using a glass pipette containing 100  $\mu$ M DA and/or 1003 receptor-specific antagonist positioned above the sensor-expressing cell. The yellow line indicates the area for line 1004 scanning. The dash lines indicate the pipette. Scale bar, 20  $\mu$ m.

1005 **b**, Representative traces showing the response measured using line-scanning; when indicated, DA and receptor-

- 1006 specific antagonist were puffed onto the cell. The trace were the average of 3 different ROIs on the scanning line.
- 1007 Data are shown as mean $\pm$ SD. Each trace was fitted with a single-exponential function to determine the  $\tau_{on}$  (left) and 1008  $\tau_{off}$  (right).

c, Group summary of τ<sub>on</sub> and τ<sub>off</sub>. τ<sub>on</sub>, *n*= 11, 8, 11, 6, 9, 8 cells for gDA3m, gDA3h, rDA2m, rDA2h, rDA3m, rDA3h. τ<sub>off</sub>,
 *n*=10, 14, 9, 7, 10, 6 cells for gDA3m, gDA3h, rDA2m, rDA2h, rDA3m, rDA3h.

1011

#### Supplementary Table 1: Properties of genetically encoded GPCR-based dopamine sensors

Sonsor	Color	CPCP backbong	Maximal	Response	Apparent affinity	On kinetics	Off kinetics	1-photon	Blue-light	Sourco	
Sensor	COIOI	GFCR backbolle	brightness	(maximum ∆F/F₀)	(EC <sub>50</sub> , nM)	(tau, ms)	(tau, s)	Ex./Em. (nm)	photoactivated $\Delta F/F_0$	Source	
gDA2m	G	D <sub>2</sub> R (human)	2.4 <sup>a,c</sup>	2.4 <sup>a</sup> 3.3 <sup>b</sup>	60ª 45 <sup>b</sup>	60*	0.71*	ND	-	ref. <sup>16</sup>	
dLight1.3b	G	D₁R (human)	1.4 <sup>a,c</sup>	3.7 <sup>a</sup> 5.2 <sup>b</sup>	870ª 690 <sup>b</sup>	ND	ND	ND	-	ref. <sup>13</sup>	
gDA3m	G	D₁R (human)	3.0 <sup>a,c</sup>	10.0ª 24.0 <sup>b</sup>	89ª 120 <sup>b</sup>	69	0.56	495/520	-	this paper	
gDA3h	G	D <sub>1</sub> R (bovine)	3.5 <sup>a,c</sup>	12.4ª 13.4 <sup>b</sup>	22ª 12 <sup>b</sup>	48	1.85	500/520	-	this paper	
rDA1m	R	D <sub>2</sub> R (human)	2.0 <sup>a,d</sup>	1.2ª 1.5 <sup>b</sup>	370ª 100 <sup>b</sup>	80*	0.77*	565/595*	-0.12	ref. <sup>16</sup>	
rDA2m	R	D <sub>2</sub> R (red fire ant)	8.2 <sup>a,d</sup>	5.3 <sup>a</sup> 6.6 <sup>b</sup>	210ª 140 <sup>b</sup>	50	2.24	565/595	-0.02	this paper	
rDA2h	R	D <sub>2</sub> R (red fire ant)	8.7 <sup>a,d</sup>	2.4 <sup>a</sup> 3.3 <sup>b</sup>	9.8 <sup>a</sup> 6.0 <sup>b</sup>	50	3.35	565/595	-0.03	this paper	
RdLight1	R	D₁R (human)	1.0 <sup>a,d</sup>	4.4 <sup>a</sup> 2.6 <sup>b</sup>	2700 <sup>a</sup> 310 <sup>b</sup>	ND	ND	560/588*	0.23	ref. <sup>15</sup>	
rDA3m	R	D₁R (human)	4.3 <sup>a,d</sup>	14.6 <sup>a</sup> 12.6 <sup>b</sup>	140ª 40 <sup>b</sup>	64	0.61	560/595	0.19	this paper	
rDA3h	R	D₁R (human)	3.8 <sup>a,d</sup>	14.2ª 10.1 <sup>b</sup>	22 <sup>a</sup> 5.5 <sup>b</sup>	60	3.60	565/585	0.20	this paper	

G: green; R: red; Ex: excitation wavelength; Em: emission wavelength; ND: not determined; <sup>a</sup> determined in HEK293T cells; <sup>b</sup> determined in cultured neurons; <sup>c</sup> relative to basal brightness of gDA2m; <sup>d</sup> relative to basal brightness of rDA1m; \* previously reported results; kinetics was estimated in HEK293T cells to puff ligand application; photoactivation was estimated in HEK293T cells with 488-nm illumination in ligand-free condition.

Note: All data were collected in this paper unless indicated.

# Extended Data Fig. 7 | gGRAB<sub>DA3h</sub> sensors report optogenetically-elicited DA release in the mouse mPFC



- 1012 Extended Data Fig. 7 | gGRAB<sub>DA3h</sub> sensors report optogenetically-elicited DA release in the mouse mPFC
- 1013 **a**, Schematic illustration depicting the experimental design for panel **b**-g.
- 1014 **b**, Representative fluorescence changes and zoom-in view (indicated by dashed box) of indicated sensors during 1015 optogenetic stimulations under control condition or in the presence of SCH-23390 (SCH).
- 1016 c, Average traces of the change in gDA3h (top) or dLight1.3b (bottom) fluorescence from a mouse. Data are shown
   1017 as mean±SD.
- 1018 **d**, Group summary of  $\Delta F/F_0$  for the indicated sensors. *n*=4 mice for gDA3h and dLight1.3b, respectively. One-way
- 1019 ANOVA, post hoc Tukey's test was performed. \*\*p=0.0035 for gDA3h; n.s. p=0.9122 for dLight1.3b; \*p=0.0295 1020 between gDA3h and dLight1.3b.
- e-f. Example fluorescence response (e) and corresponding average traces (f) of gDA3h (top) or dLight1.3b (bottom)
   to indicated optogenetic stimulation. The average traces are shown as mean±SD.
- 1023 **g**, Group summary of peak  $\Delta F/F_0$  of gDA3h or dLight1.3b in response to indicated optogenetic stimulation. n=4 mice 1024 for gDA3h and dLight1.3b.
- 1025

# Extended Data Fig. 8 | rGRAB sensors report optogenetically-elicited DA release in multiple brain regions *in vivo*



- 1026 Extended Data Fig. 8 | rGRAB sensors report optogenetically-elicited DA release in multiple brain regions *in vivo*
- 1027 **a**, Schematic illustration depicting the experimental design for panel **b-d**.
- b, Representative traces of rDA3m or rDA3mut signals during optogenetic stimulations. rDA3m signals were
   measured before and after SCH-23390 (SCH) administration.
- c, Average traces of the change in sensor fluorescence to 1-, 5- or 10-s opto-stimulation from a mouse. Data are
   shown as mean±SD. The blue shaded area indicates the application of opto-stimulation.
- **d**, Group summary of peak response of rDA3m or rDA3mut to indicated optogenetic stimulation. *n*=3 mice for rDA3m and *n*=5 for rDA3mut. Two-tailed Student's t-test was performed. p=0.0278, 0.0101, 0.0068 between control and SCH to 1-, 5-, 10-s opto-stimulation. p=0.0003, 0.0001, <0.0001 between rDA3m and rDA3mut to 1-, 5-, 10-s opto-stimulation.
- 1036 **e**, Schematic illustration depicting the experimental design for panel **e-h**.
- 1037 **f**, Representative traces of sensor signals simultaneously recorded in the mPFC (top) and NAc (bottom) during 1038 optogenetic stimulations.
- 1039 **g**, Average traces of the change in sensor fluorescence in the mPFC (left) and NAc (right) to indicated optogenetic 1040 stimulation from a mouse. Data are shown as mean±SD. The length of blue lines indicates the duration of opto-1041 stimulation.
- 1042 **h**, Group summary of peak response of rDA2m or rDA2mut to indicated optogenetic stimulation. *n*=3 mice for
- 1043 rDA2m and rDA2mut. Two-tailed Student's t-test was performed between rDA3m and rDA3mut response upon 10-1044 s opto-stimulation. p=0.0007 for mPFC, p=0.0364 for NAc.
- 1045

# Extended data Fig. 9 *In vivo* comparison of the third-generation DA sensors versus previous variants in water-restricted mice receiving water rewards.



1046 Extended data Fig. 9 | In vivo comparison of the third-generation DA sensors versus previous variants in water-

### 1047 restricted mice receiving water rewards

- 1048 **a**, Diagram of mouse surgical procedure. AAVs carrying gGRAB<sub>DA2m</sub>, gGRAB<sub>DA3m</sub>, rGRAB<sub>DA1m</sub>, or rGRAB<sub>DA3m</sub> were 1049 injected unilaterally into NAc. An optic fiber was implanted above the injection site.
- 1050 **b**, Illustration of behavioral experiment.
- 1051 **c**, Recording sessions from gDA2m mice. Vertical black bars indicate water delivery. Colors indicate water volume.
- 1052 **d**, Recording sessions from gDA3m mice.
- 1053 **e**, Peak response to 8  $\mu$ L water for the sessions shown in **c** and **d**. \*\* p = 0.0095, Mann-Whitney U test.
- 1054 **f**, Recording sessions from rDA1m mice.
- 1055 g, Recording sessions from rDA3m mice.
- 1056 **h**, Peak response to 8  $\mu$ L water for the sessions shown in **f** and **g**. \*\* p = 0.0286, Mann-Whitney U test.
- 1057 **i-j**, Schematic illustration depicting the mouse surgical procedure and the experimental design for panel **k-l**.
- 1058 **k**, Recording sessions from 3 mice. Vertical black bars indicate water delivery. Colors indicate sensor version.
- 1059 I, Peak response of rDA3m and RdLight1 for the sessions shown in k. \* p = 0.0249, Two-tailed Student's t-test.

# Extended data Fig. 10 | The signals in the mouse NAc and mPFC during Pavlovian conditioning



#### 1060 Extended data Fig. 10 | The signals in the mouse NAc and mPFC during Pavlovian conditioning

- 1061 **a**, Representative fluorescence signals recorded during consecutive water trials pre (top, control) and post SCH-
- 1062 **23390** (bottom, SCH-23390) treatment. The audio and water delivery are indicated above.
- b, Averaged traces of rDA3m (left) and ACh3.0 (right) fluorescence measured in the NAc from a mouse under control
   condition or in the presence of SCH-23390. Shown are more than 50 consecutive trials (mean±SD) in one mouse.
- 1065 The grey shaded area indicates the application of audio. The dashed line indicates the delivery of water.
- 1066 **c**, Group summary of the peak fluorescence change of rDA3m and ACh3.0 signals in the NAc under the indicated
- 1067 condition. *n*= 155 trials from 3 mice for each group. Two-tailed Student's t-test was performed between control and
- 1068 SCH-23390 group. p=0.2624 for ACh3.0.
- 1069 **d-e**. same as (**b-c**) with simultaneously recorded rDA3m and ACh3.0 signals in the mPFC. Two-tailed Student's t-test
- 1070 was performed between control and SCH-23390 group. p=0.2274 for ACh3.0.
- 1071

# Extended data Fig. 11 | In vivo two-photon imaging of cortical DA dynamics in mice



## 1072 Extended data Fig. 11 | *In vivo* two-photon imaging of cortical DA dynamics in mice

- 1073 **a-b**, Schematic illustration depicting the experimental design for panel **c-j**.
- 1074 c-e, Representative expression and pseudocolored response images (c), representative traces measured at the
   1075 indicated ROIs (d), and average traces per forced running (e) measured in the head-fixed mice expressing gDA3m,
   1076 gDA3h, dLight1.3b or mEGFP. Scale bar, 100 μm.
- 1077 f, Group summary of the peak fluorescence response (top) and SNR (bottom) measured during forced running in the motor cortex of mice expressing gDA3m, gDA3h, dLight1.3b and mEGFP. n=14 trials from 4 mice (15/4) for 1078 gDA3m, 13/4 for gDA3h, 9/3 for dLight1.3b, 12/4 for mEGFP. Paired two-tailed Student's t-test was performed within 1079 group. One-way ANOVA, post hoc Tukey's test was performed across sensor groups. Response, p<0.0001 for gDA3m; 1080 p=0.0002, 0.0683, 0.6275 for gDA3h, dLight1.3b, mEGFP; p<0.0001 between gDA3h and dLight1.3b, or EGFP; 1081 p=0.0214 between gDA3m and dLight1.3b; p=0.0022 between gDA3m and mEGFP; p=0.1611 between gDA3m and 1082 gDA3h; p=0.9577 between dLight1.3b and mEGFP. SNR, p<0.0001 between gDA3h and mEGFP; p=0.0004 between 1083 gDA3h and dLight1.3b; p=0.0016 between gDA3m and mEGFP; p=0.0337 between gDA3m and dLight1.3b; p=0.8812 1084 1085 between dLight1.3b and mEGFP.
- 1086 **g**, Summary of the rise and decay  $t_{50}$  values (where applicable) of the gDA3m and gDA3h signals in response to 1087 forced running.
- 1088 **h-j**, Same as (**c-e**) except mice were subjected to tail shock.
- 1089 **k**, Group summary of the peak fluorescence response (top) and SNR (bottom) measured upon tail shock in the motor
- 1090 cortex of mice expressing gDA3m, gDA3h, dLight1.3b and mEGFP. n=19/4 for gDA3m, 16/4 for gDA3h, 12/3 for
- 1091 dLight1.3b, 26/4 for mEGFP. Paired two-tailed Student's t-test was performed within group. One-way ANOVA, post 1092 hoc Tukey's test was performed across sensor groups. Response, p<0.0001 for gDA3m and gDA3h; p=0.1774, 0.2524
- 1093 for dLight1.3b, mEGFP; p<0.0001 between mEGFP and gDA3m, or gDA3h; p<0.0001 between gDA3h and dLight1.3b;
- 1094 p=0.0.0013 between gDA3m and dLight1.3b; p=0.7169 between gDA3m and gDA3h; p=0.3714 between dLight1.3b
- 1095and mEGFP. SNR, p<0.0001 between mEGFP and gDA3m, or gDA3h; p=0.0186, 0.0104 between dLight1.3b and</th>1096gDA3m, or gDA3h; p=0.2607 between dLight1.3b and mEGFP.
- 1097 I, Summary of the rise and decay t<sub>50</sub> values of the gDA3m and gDA3h signals in response to tail shock. mEGFP data
   1098 replotted from Fig. 6f.