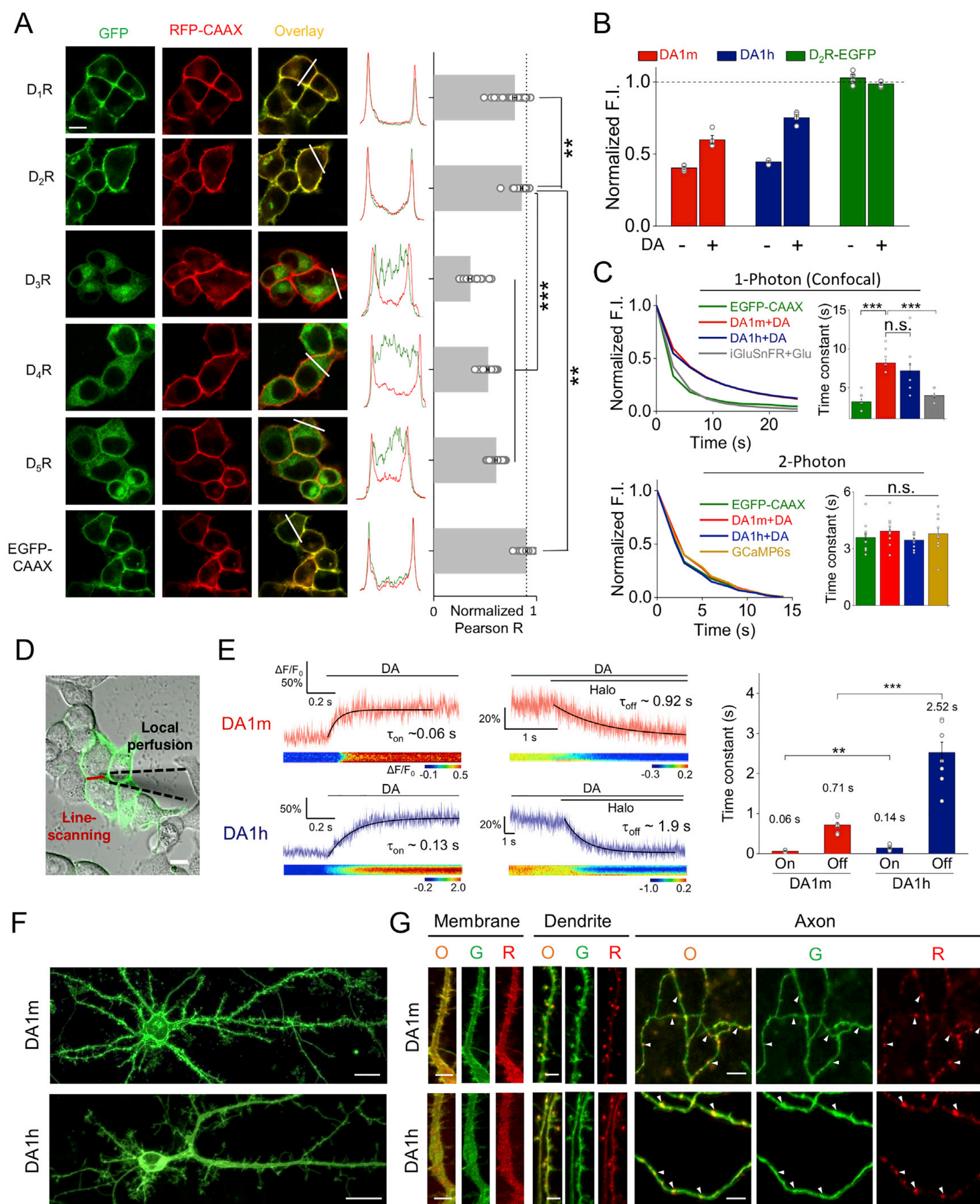

Figure S1. Screening and Characterization of $GRAB_{DA}$ Sensors in HEK293T Cells or Neurons, Related to Figure 1

(A) The fluorescence and membrane trafficking of all five DRs with cpEGFP insertion. A membrane-localized RFP (RFP-CAAX) was co-expressed to indicate the plasma membrane and EGFP-CAAX was used as a control. Left, the fluorescence images of HEK293T cells expressing all five DR-based chimeras or EGFP-CAAX (green) and RFP-CAAX (red); scale bar, 10 μM; white bars in overlay panels, line-scanning region. Middle, the normalized line-scanning plots of the fluorescence signals in both green and red channels. Right, Pearson's colocalization ratios of the DR-based chimeras or EGFP-CAAX according to RFP-CAAX (n = 30/2 for each protein; $p < 0.001$ comparing D_2R with D_3R, D_4R and D_5R; $p = 0.001$ between D_2R and EGFP-CAAX; $p = 0.006$ between D_2R and D_1R).

(B) Relative brightness of DA1m and DA1h compared with D_2R-EGFP chimeric construct in the absence and presence of 100 μM DA (n = 4 wells/group with 100-200 cells/well).

(C) Photostability of DA1m and DA1h compared with other fluorescent probes. Top: Representative photobleaching curves of DA1m, DA1h, EGFP-CAAX and iGluSnFR expressed in HEK293T cells under confocal imaging (488 nm laser with the laser power of ~350 μW and the intensity of ~1.75×10^2 W/cm^2) are shown in left. The group data of decay time constants of each are shown in right (n = 10/3 of each group; $p = 0.350$ between DA1m and DA1h; $p < 0.001$ comparing DA1m

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with EGFP-CAAX and iGluSnFR). Bottom: Similar as top, except that two-photon laser (920nm laser with the laser power of ~27.5 mW and the intensity of ~1.38×10^4 W/cm^2) was used to test the photostability of DA1m, DA1h, EGFP-CAAX and GCaMP6s (n = 12/2 of each group; $p = 0.251$ between EGFP-CAAX and DA1m; $p = 0.583$ between EGFP-CAAX and DA1h; $p = 0.537$ between EGFP-CAAX and GCaMP6s; $p = 0.051$ between DA1m and DA1h; $p = 0.678$ between DA1m and GCaMP6s; $p = 0.236$ between DA1h and GCaMP6s).

(D) Schematic image showing the rapid local perfusion system. A glass pipette (black dashed lines) filled with DA or Halo was positioned close to the $GRAB_{DA}$-expressing cells, and fluorescence signals were measured using confocal line-scanning (red line).

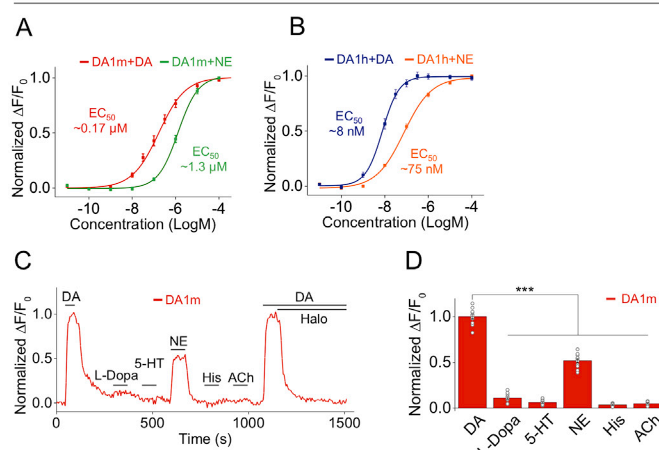
(E) Left and middle: Representative fluorescence changes in $GRAB_{DA}$-expressing cells in response to the local perfusion (on rate: 100 μM DA in pipette with normal bath solution; off rate: 1 mM Halo in pipette with bath solution containing 10 μM DA for DA1m or 1 μM DA for DA1h). The traces are the average of 3 different ROIs on the scanning line, shaded with ± SEM and fitted with a single-exponential function (black traces, with the decay time constants shown). Right: Group data summarizing the response kinetics of $GRAB_{DA}$-expressing cells in response to DA (on) or Halo (off) (n = 8 cells/group; $p = 0.0093$ between on kinetics; $p < 0.001$ between off kinetics).

(F) Expression of $GRAB_{DA}$ sensors in cultured neurons. Scale bars, 20 μm.

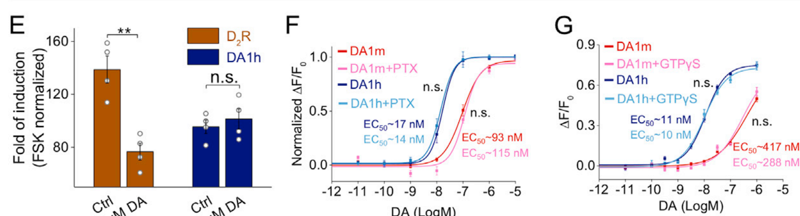
(G) Expression and localization of $GRAB_{DA}$ sensors (green, G), subcellular markers (red, R) and the overlay (O) in the indicated subcellular compartments in cultured neurons. RFP-CAAX, PSD95-RFP and Synaptophysin-RFP were co-expressed as markers of the plasma membrane, dendritic spines, and presynaptic boutons, respectively. Arrow heads indicate synaptic boutons. Scale bars, 5 μm.

Values with error bars indicate mean ± SEM. Student's t test performed; n.s., not significant; $^{**}p < 0.01$; $^{***}p < 0.001$.

Selectivity on cultured neurons



G protein-dependent signaling



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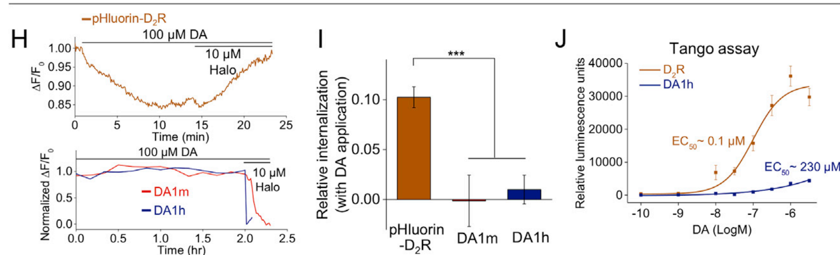

Figure S2. Selectivity to DA and NE, as well as the Efficacy Coupling Downstream Signaling of DA1m/h-Expressing HEK293T Cells or Cultured Neurons, related to Figure 1

(A and B) Normalized dose-dependent fluorescence responses to DA and NE of DA1m- (A) or DA1h- (B) expressing cultured neurons (DA1m+DA: n = 10/6; DA1m+NE: n = 12/5; DA1h+DA: n = 10/5; DA1h+NE: n = 11/8).

(C and D) Representative trace (C) and group analysis (D) of the fluorescence changes of DA1m-expressing neurons in response to the sequential application of the indicated compounds at 1 μM, including DA, L-Dopa, 5-HT, NE, His, ACh, DA(2nd) and DA+Halo (n = 12/12; $p < 0.001$ comparing responses to DA with L-Dopa, 5-HT, NE, His, ACh and DA+Halo).

(E) Fold of induction, which revealed the cAMP level, in the luciferase assay of D_2R- (brown) or DA1h- (blue) expressing Nanoluc reporter cells bathed in control normal solution or solution containing 100 μM DA (n = 4 wells/group; p = 0.002 for D_2R; p = 0.544 for DA1h).

(F) Normalized fluorescence changes in $GRAB_{DA}$-expressing cells in response to the application of DA, with or without the co-expression of pertussis toxin (PTX) (DA1m: n = 14/3; DA1m+PTX: n = 14/3; DA1h: n = 10/3; DA1h+PTX: n = 10/3; p = 0.680 comparing the EC_{50} of DA1m and DA1m+PTX; p = 0.810 comparing the EC_{50} of DA1h and DA1h +PTX).

(G) Fluorescence changes in $GRAB_{DA}$-expressing cells in response to the application of DA, with or without the pre-bathing of GTPγS (n = 3 well/group with 100-300 cell/well; p = 0.488 comparing the EC_{50} of DA1m and DA1m+GTPγS; p = 0.474 comparing the EC_{50} of DA1h and DA1h+GTPγS).

(H) Top: The representative trace of the total fluorescence changes in pHluorin-D_2R-expressing HeLa cells in response to the application of 100 μM DA followed by 10 μM Halo. Bottom: Exemplar traces of normalized fluorescence changes of DA1m- (red) and DA1h- (blue) expressing neurons during a 2-hour application of 100 μM DA.

(I) Quantification of relative internalization of pHluorin-D_2R, DA1m and DA1h in response to the application of 100 μM DA (pHluorin-D_2R: n = 12/2; DA1m: n = 20/12; DA1h: n = 14/6).

(J) Dose-dependent luminescence units in the tango assay of D_2R- (brown) or DA1h- (blue) expressing cells in response to DA application (n = 3 well/group with 100-300 cell/well).

Values with error bars indicate mean ± SEM. Student's t test performed; n.s., not significant; **$p < 0.01$; ***$p < 0.001$.

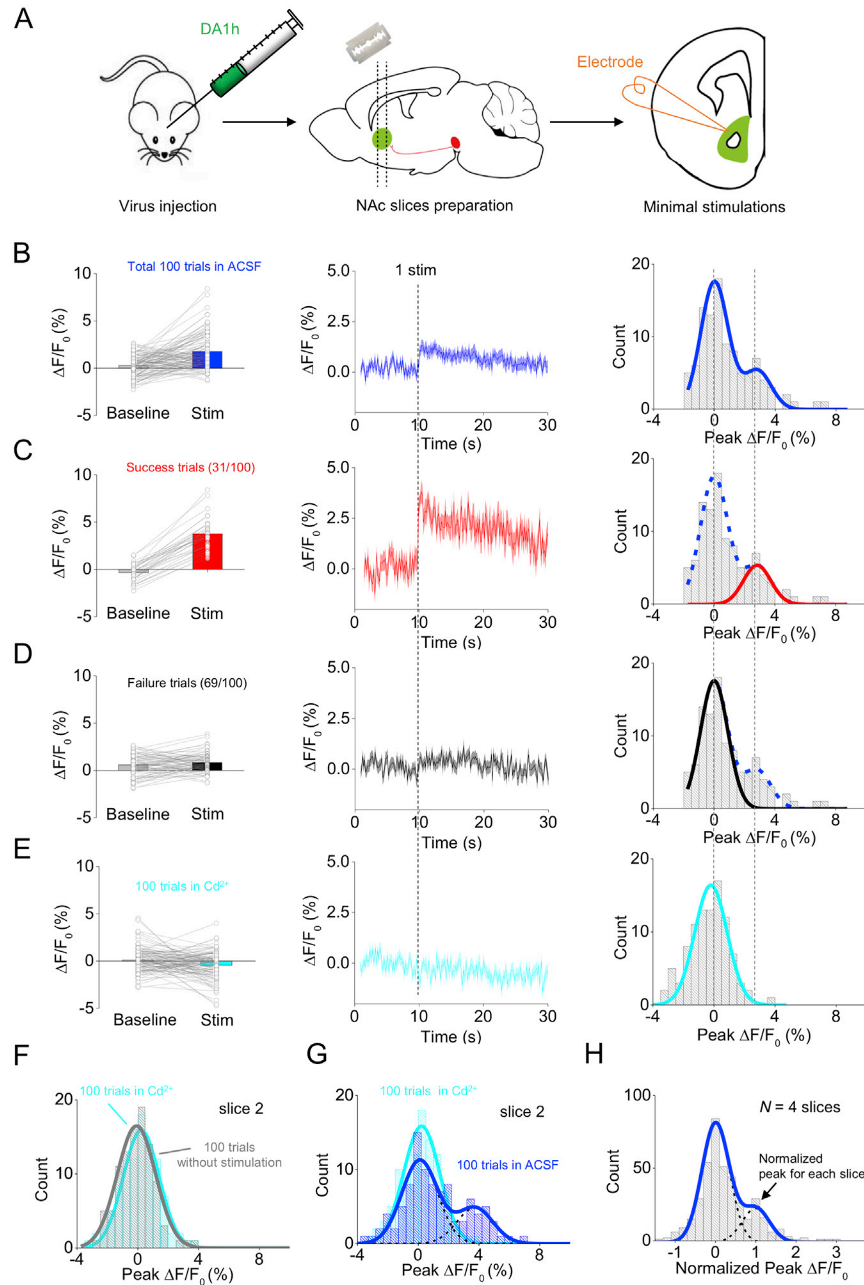

Figure S3. GRAB$_{DA}$ Sensors Report Minimal Stimulation-Induced DA Release in NAc Slices, Related to Figure 2

(A) Schematic illustration of the DA1h virus injection, NAc slice preparation and minimal electrical stimulation in mice.

(B–D) Left, the fluorescence responses of DA1h-expressing neurons before (baseline) and after stimulation (stim) in a total of 100 trials under minimal stimulation condition (B), of which 31 trials were successes (C) and 69 trials were failures (D). The $\Delta F/F_0$ of 3 frames before stimulation and immediately after stimulation were averaged as "baseline" and "stim." Middle, the averaged fluorescence traces of all 100 trials (B), of 31 success trials (C), of 69 failure trials (D). Right, the distribution of peak $\Delta F/F_0$ of all 100 trials (B, blue), with success trials highlighted in (C, red) and failure trials highlighted in (D, black).

(E) Similar as (B), except that ACSF containing 200 μM Cd^{2+} was bathed to the same slice.

(F) A different DA1h- expressing slice where distributions of peak $\Delta F/F_0$ in 100 minimal stimulation trials in ACSF (gray) and in ACSF containing Cd^{2+} (light blue) were compared.

(G) Similar as (F), except the distributions of peak $\Delta F/F_0$ in ACSF (blue) and ACSF containing Cd^{2+} (light blue) were compared.

(H) Distribution of the group data under minimal stimulation, where peak $\Delta F/F_0$ from individual DA1h- expressing slices was normalized and pooled together (~400 stimulation trials in total from 4 slices of 3 mice). Note, the normalization was done by first fitting for each set of data to identify the peak of failures as "zero" and peak of success as "one".

Values with error bars indicate mean ± SEM.

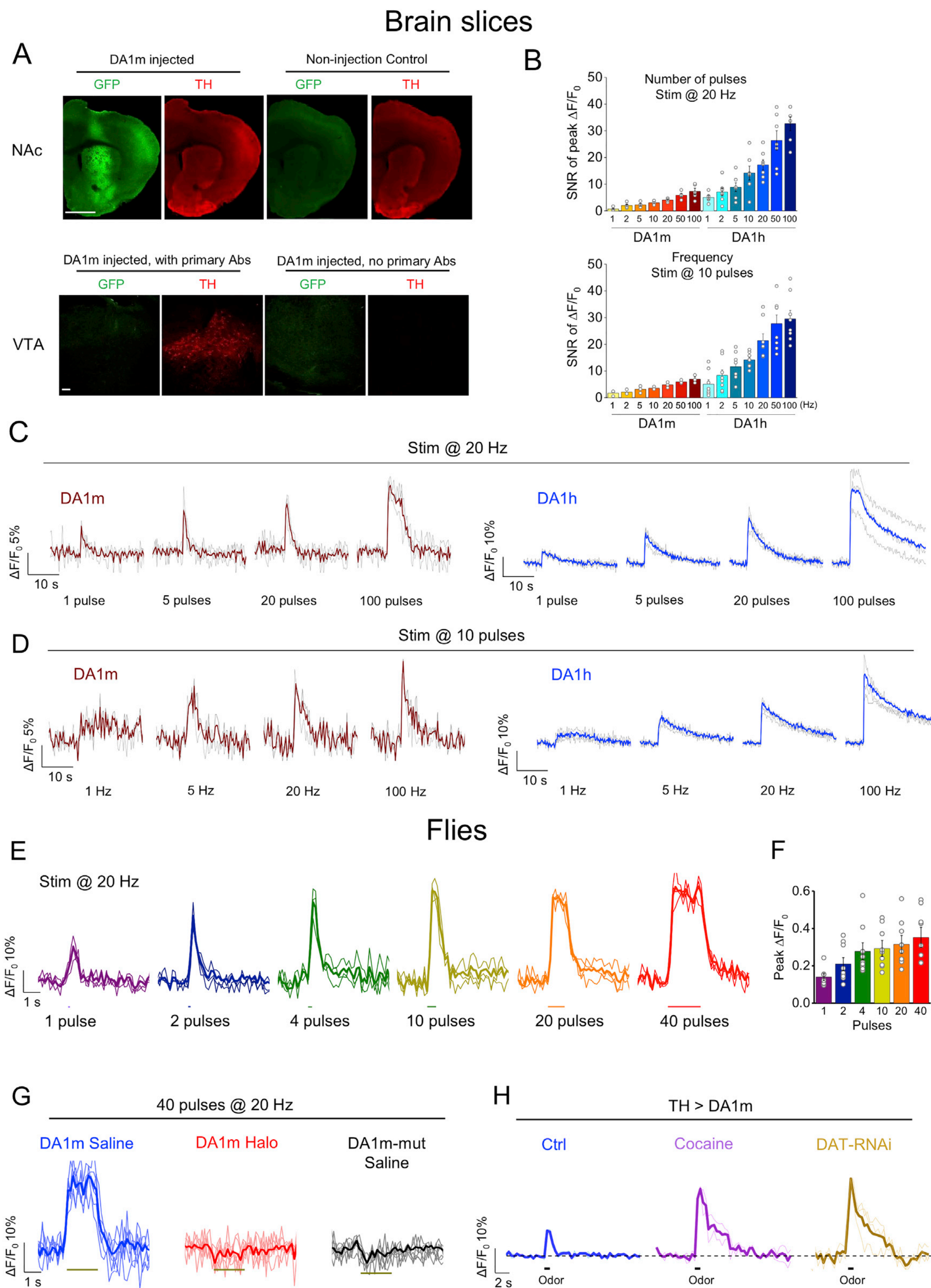

Figure S4. The Single-Trial Data of DA1m Fluorescence Responses in NAc Brain Slices and Flies, Related to Figures 2 and 3

(A) Top: representative immunoreactive signals of GFP (green) and TH (red) in NAc slices of DA1m injected mice (left) or non-injection control mice (right). Scale bar, 1mm.

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Bottom: representative immunoreactive signals of GFP (green) and TH (red) in VTA slices of DA1m injected mice with (left) or without (right) the application of primary antibodies. Scale bar, 10 μm.

(B) Group analysis of the signal-to-noise ratio (SNR) of the fluorescence responses of DA1m- or DA1h-expressing neurons to a trial of electrical stimuli at different pulse numbers (left, n = 5 slices from 3 mice in DA1m; n = 7 slices from 4 mice in DA1h), or a trial of 10-pulse electrical stimuli with different frequencies (right, n = 3 slices from 2 mice in DA1m; n = 5-8 slices from 3 mice in DA1h).

(C) Three single trials (gray) and corresponding averaged trials (red in DA1m, blue in DA1h) of fluorescence responses of DA1m- or DA1h-expressing neurons to a train of 20-Hz electrical stimuli containing the indicated pulse numbers.

(D) Similar as in (C) except that a train of 10-pulse electrical stimuli at the indicated frequencies was applied.

(E and F) Fluorescence changes of TH > DA1m flies in response to electrical stimuli with indicated pulses at 20 Hz. Representative 3 single trial traces (light) with corresponding averaged traces (bold) from one fly are shown in (E). Group data of peak $\Delta F/F_0$ are summarized in (F) (n = 9 flies/group).

(G) Fluorescence changes of TH > DA1m and TH > DA1m-mut flies in response to 40-pulse electrical stimuli at 20 Hz, in normal saline or in saline containing 10 μM Halo. Representative 6 single trial traces (light) with corresponding averaged traces (bold) from one fly/group are shown.

(H) Fluorescence changes of TH > DA1m flies in response to 1-s odor stimulation in control condition (left), in the presence of the DAT blocker cocaine (3 μM, middle), or in DAT-RNAi genetic background (right). Representative 2-3 single trial traces (light) with corresponding averaged traces (bold) from one fly/group are shown.

Values with error bars indicate mean ± SEM.

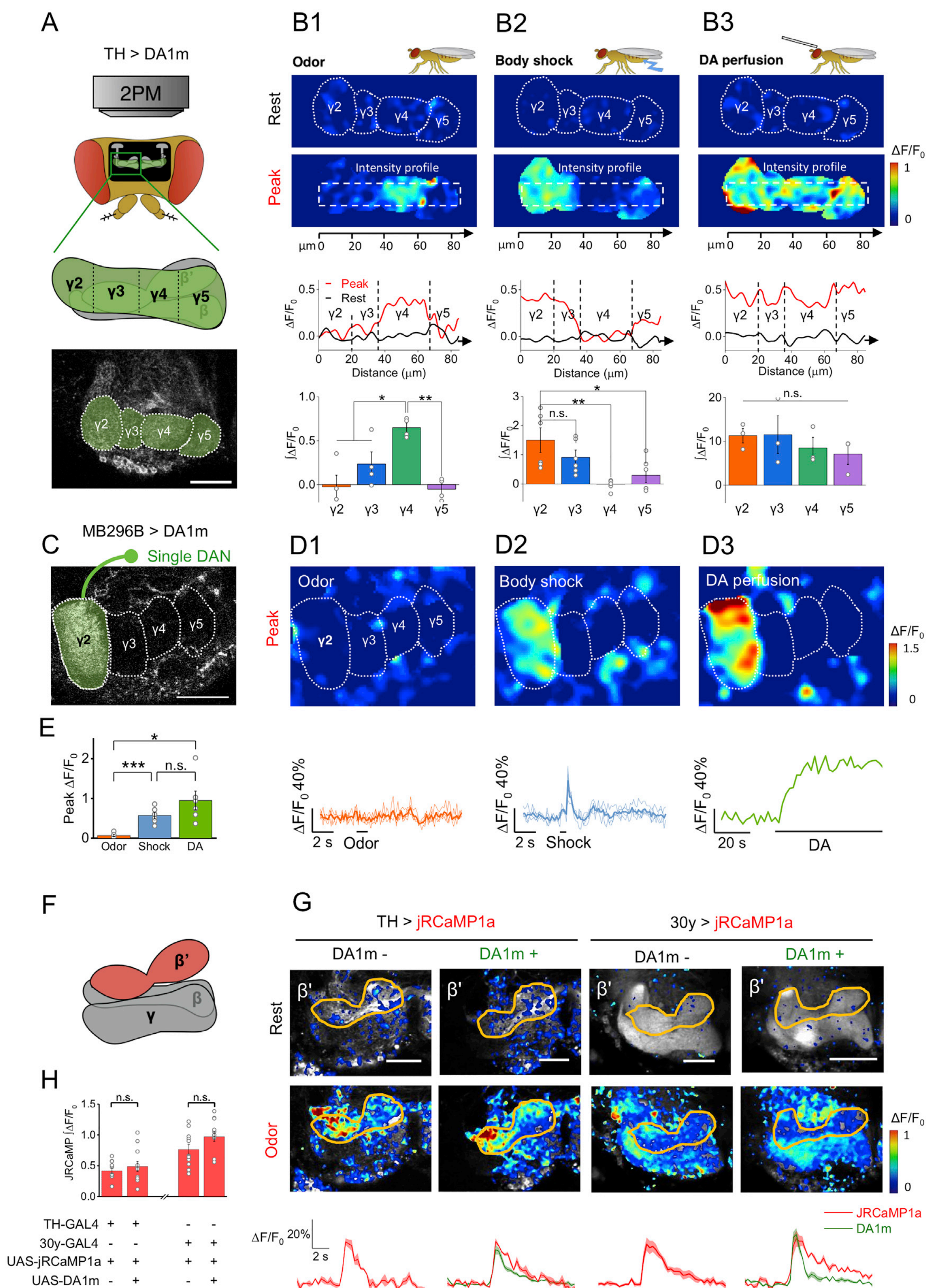

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Figure S5. Characterization of the Spatial Resolution and Sensitivity and the Effect on Ca^{2+} Signaling of DA1m by Fly *In Vivo* Imaging, Related to Figure 3

(A) Top panel, schematic illustration showing the TH > DA1m fly *in vivo* two-photon imaging. Bottom panels, the cartoon and the field of view indicate MB γ2-5 compartments in this focal plane.

(B) Fluorescence signals of TH > DA1m fly in response to 1-s odor, 500-ms body shock and 100 μM DA perfusion. Representative pseudocolor images and corresponding intensity profiles from one fly are shown in top and middle panels. Group data are summarized in bottom panels (n = 4 flies for odor; n = 6 flies for body shock; n = 3 flies for DA perfusion; for odor, comparing γ4 with γ2, γ3 and γ5, p = 0.011, 0.027 and 0.002; for body shock, comparing γ2 with γ3, γ4 and γ5, p = 0.211, 0.009 and 0.027; for DA perfusion, p = 0.967 between γ2 and γ3, p = 0.284 between γ2 and γ4; p = 0.305 between γ2 and γ5; p = 0.131 between γ3 and γ4; p = 0.282 between γ3 and γ5; p = 0.944 between γ4 and γ5).

(C) Schematic illustration and the field of view depicting the expression of DA1m in single DAN each hemisphere driven by MB296B-GAL4.

(D and E) Fluorescence signals of MB296B > DA1m fly in response to 1-s odor, 500-ms body shock and 100 μM DA perfusion. Representative pseudocolor images (averaged from 3 trials) and corresponding traces (light, 3 single trial traces; bold, 3-trial averaged traces) from one fly are shown in (D). The group analysis of peak $\Delta F/F_0$ are summarized in (E) (n = 5 flies for odor; n = 6 flies for body shock; n = 6 flies for DA perfusion; p < 0.001 between sham and body shock; p = 0.013 between sham and DA perfusion; p = 0.178 between body shock and DA perfusion).

(F) Schematic illustration of the MB β' lobe.

(G and H) Fluorescence responses of jRCaMP1a- and DA1m-exprssing DANs (left) or Kenyon cells (right) to 1-s odor stimulation. Representative pseudocolor images and corresponding 3-trial-averaged traces shaded with ± SEM from one fly are shown in (G). Group data of the integrals of jRCaMP1a signals are summarized in (H) (TH > jRCaMP1a: n = 10 flies; TH > jRCaMP1a, DA1m: n = 11 flies; 30y > jRCaMP1a: n=11 flies; 30y > jRCaMP1a, $GRAB_{DA1m}$: n = 12 flies; p = 0.503 between TH > jRCaMP1a and TH > jRCaMP1a, DA1m; p = 0.097 between 30y > jRCaMP1a and 30y > jRCaMP1a, DA1m).

Scale bars in (A), (C), and (G) are 25 μm. Values with error bars indicate mean ± SEM. Student's t test performed; n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

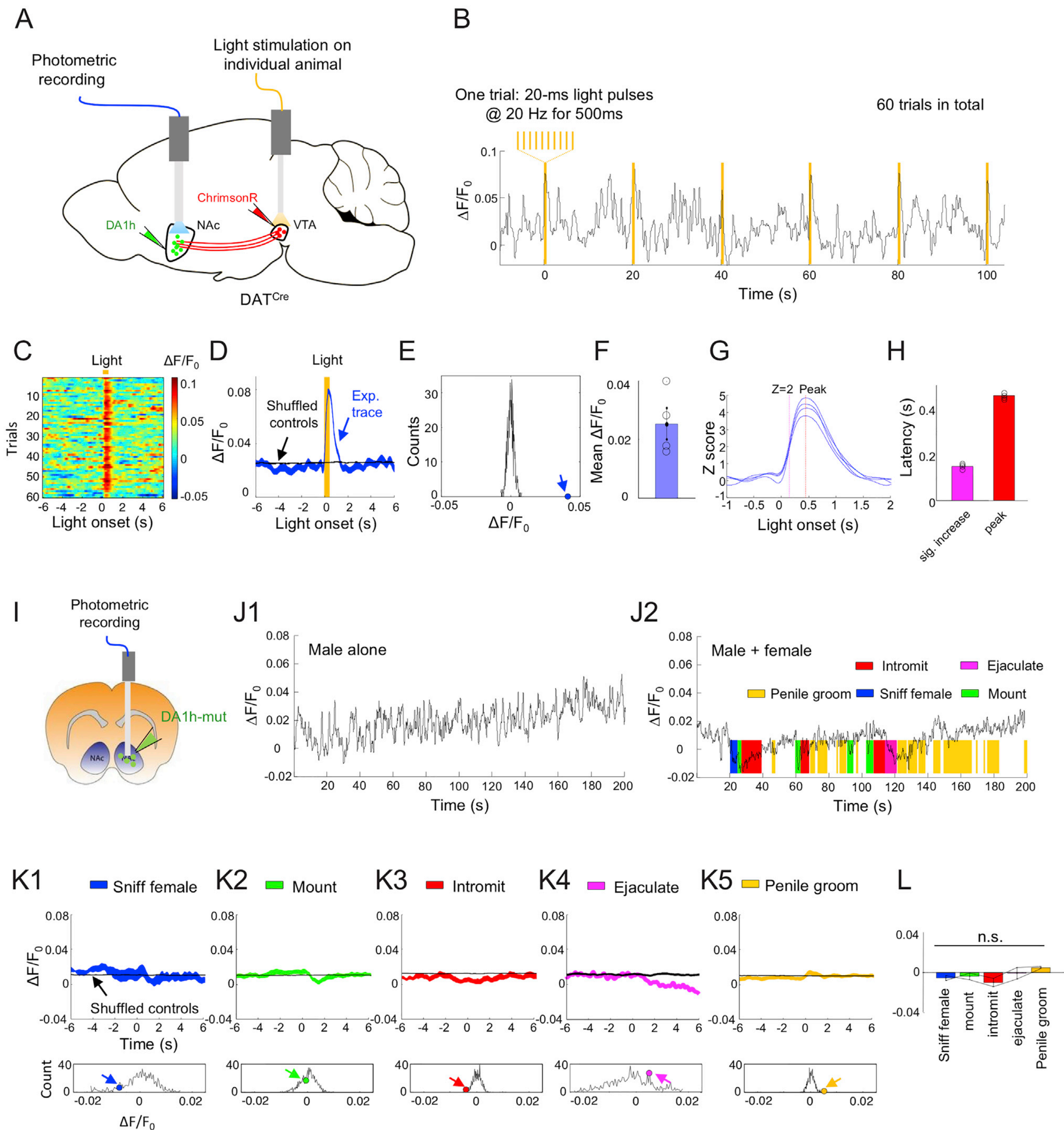

Figure S6. Fluorescence Signals of DA1h in NAc Evoked by Optogenetically Stimulation of DANs in VTA and Signals of DA1h-mut in NAc during Male Sexual Behaviors, Related to Figure 7

(A) Schematic diagram showing the experimental design used to record DA1h signals in the NAc (AAV hsyn-DA1h) while optogenetically activating the VTA DANs (AAV hsyn-Flex-ChrimsonR-tdTomato). 593-nm 20-ms yellow light was delivered at 20 Hz for 500 ms in each trial, and 60 trials were applied with 20-s interval to one animal.

(B–E) Representative fluorescence changes during optogenetic stimulations from one mouse. (B) Snapshot of 6 trials. (C) Heatmap showing all 60-trial fluorescence signals aligned to the light onset. (D) Post-event histogram (PETH) showing the averaged fluorescence changes of 60-trial light stimulation (blue) and 1000 × shuffled controls (black). Shades represent ± SEM. (E) The distributions of mean $\Delta F/F_0$ of shuffled controls. The colored dot and arrow indicate the actual mean $\Delta F/F_0$ during the light stimulation.

(F) Group data summarizing the mean $\Delta F/F_0$ increase during light stimulation (n = 4 animals). Error bar: ± SEM. Student t test is performed to compare the mean $\Delta F/F_0$ of stimulation with that of shuffled controls, $F(3) = 4.60$, $p = 0.019$.

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(G) Z score normalized PETHs of all 4 animals aligned to the light onset. The Z score at each time point is calculated using the −6 to 0 s prior to light onset as the baseline. Magenta line indicates the average latency to reach Z = 2. Red line indicates the average latency to reach peak response.

(H) Group data summarizing the latency to reach significant increase (Z = 2) and peak response after light stimulation.

(I) Schematic diagram showing the experimental design used to record DA1h mutant signals in the NAc (AAV hsyn-DA1h-mut) of male mice during sexual behaviors.

(J) Representative fluorescence changes right before female introduction (Ji) and during male sexual behaviors (Jii). The shaded areas with colors indicate different behavioral events.

(K) Top: PETHs showing the fluorescence changes aligned to various behavioral events. Black lines show averaged PETHs of 1000 × shuffled controls. Shades represent ± SEM. Bottom: The distributions of mean $\Delta F/F_0$ of shuffled controls. Colored dots and arrows indicate the actual mean $\Delta F/F_0$ during each behavior. Mean $\Delta F/F_0$ is calculated as averaged $\Delta F/F_0$ from 0-1 s after the behavioral onset minus the baseline $\Delta F/F_0$. Baseline for sniff, mount, intromit and lick is defined as −12 to −2 s before the onset of each behavioral episode. Baseline for ejaculate is defined as 10 to 20 s after the onset of the behavior given that ejaculation is always preceded by mounting and intromission.

(L) Group data summarizing the mean $\Delta F/F_0$ during various behaviors of two animals. One-way ANOVA with repeated-measures. Among behaviors: $F(3, 4) = 0.63$. $p = 0.473$.

Values with error bars indicate mean ± SEM.