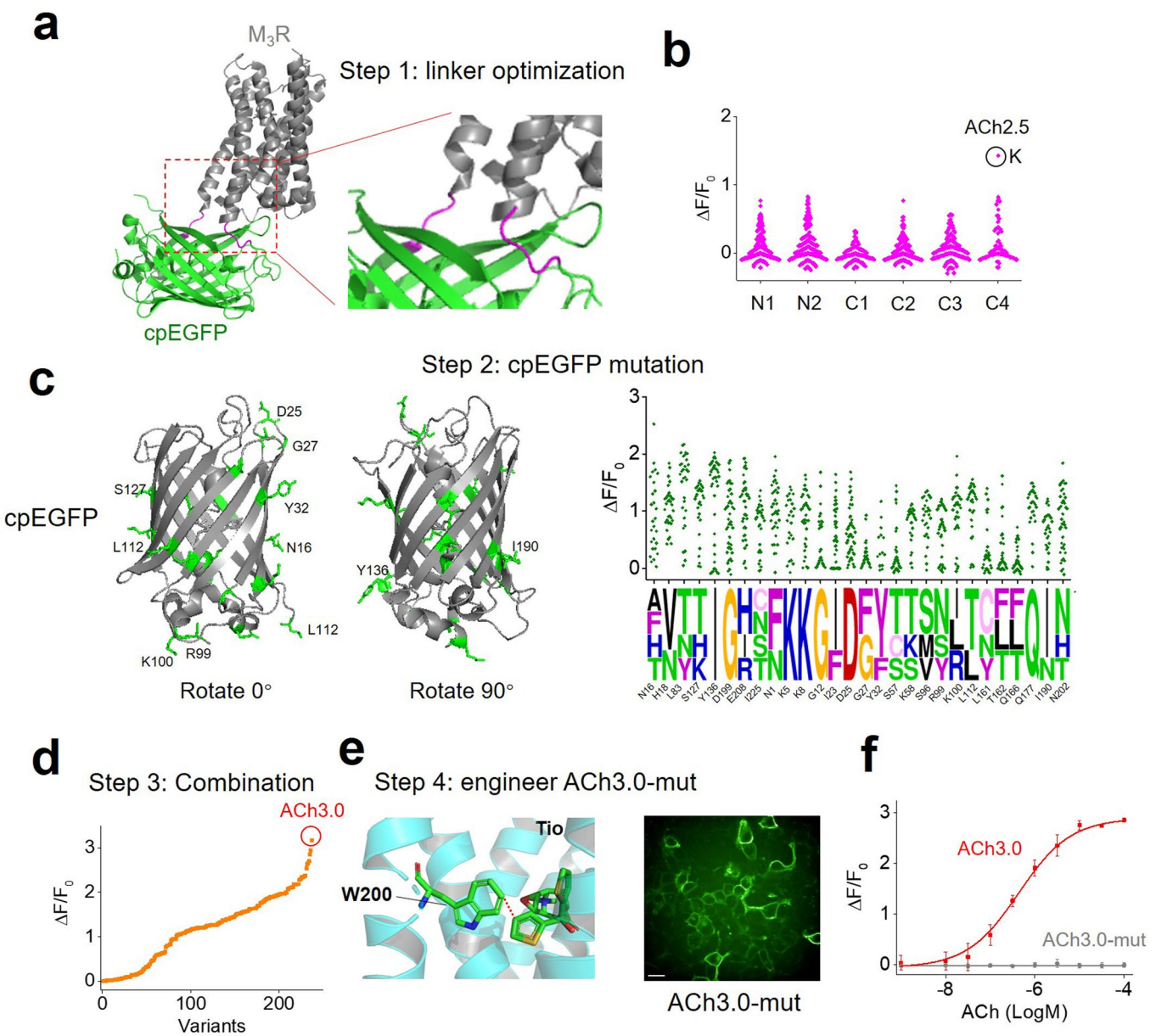
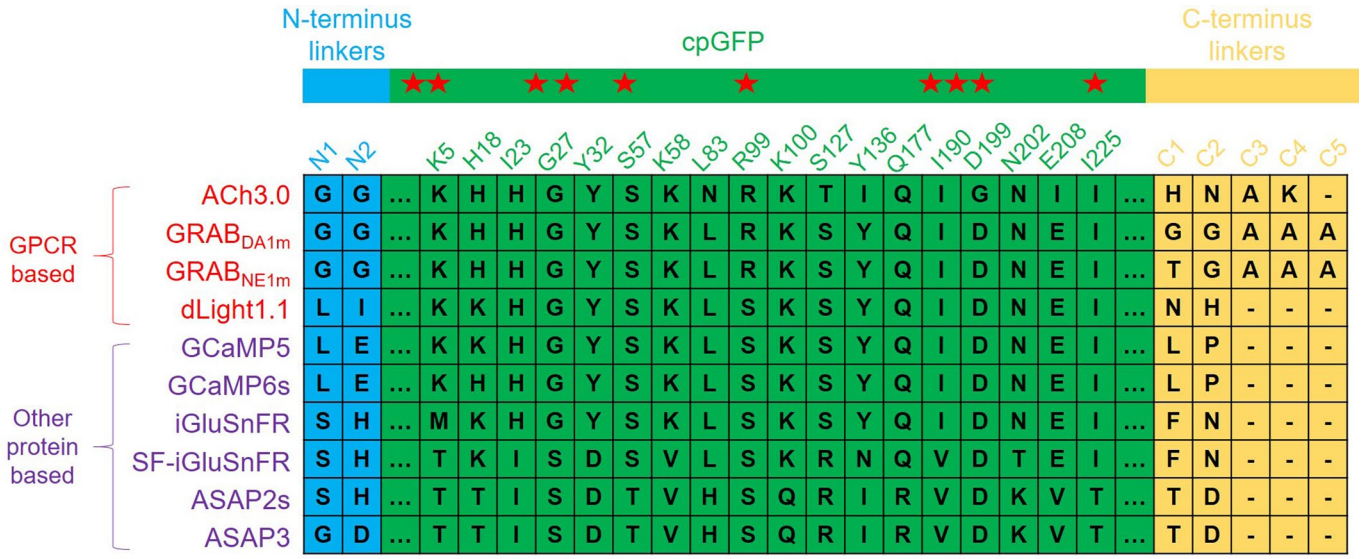
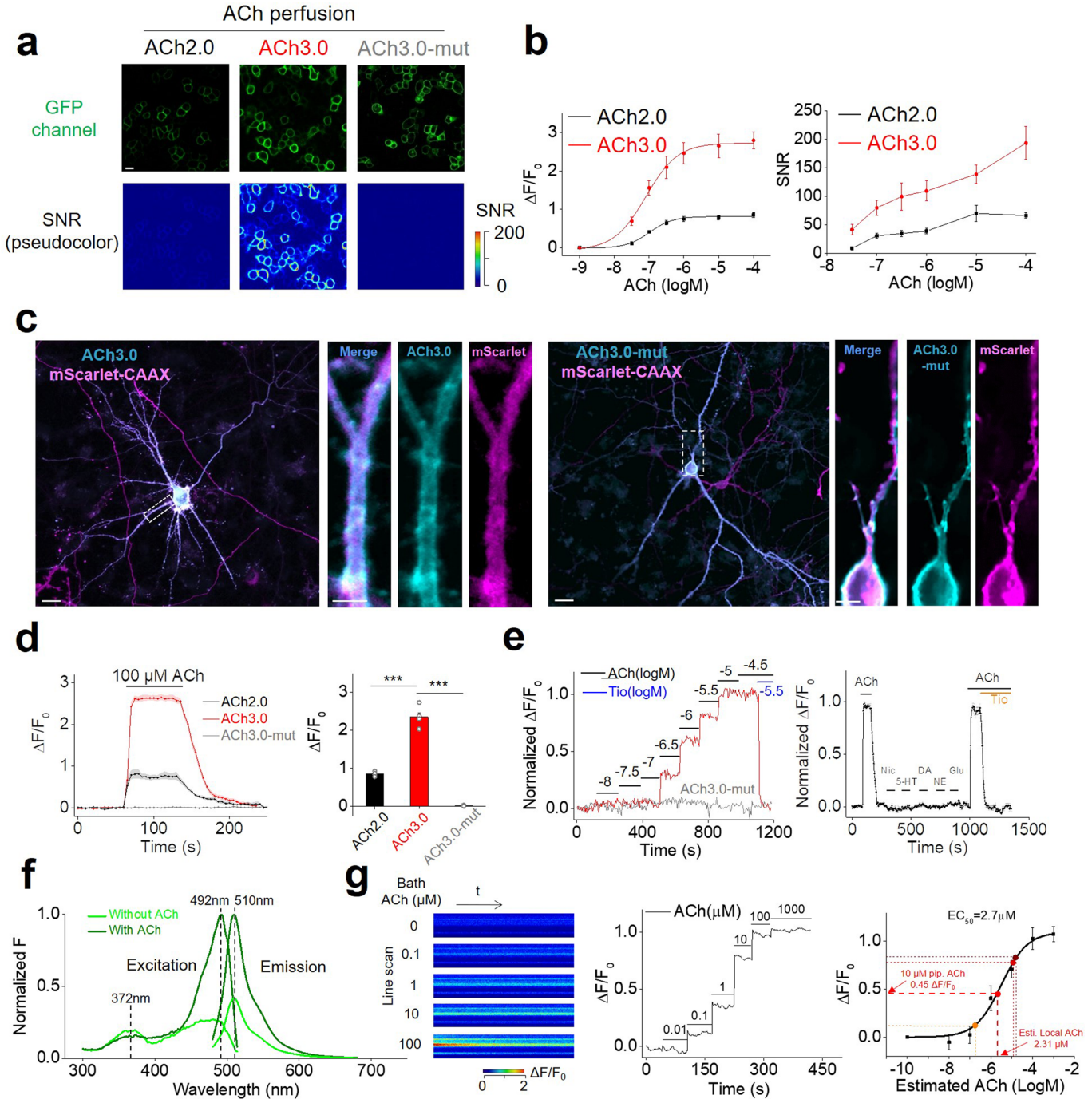


Extended Data Fig. 1 | The engineering process leading to the GRAB$_{ACh3.0}$ sensor. a, Schematic illustration depicting the predicted structure of the generic GRAB$_{ACh}$ sensor, with the linker region between the receptor (M_3R) and cpEGFP magnified at the right and shown in magenta. The crystal structures are from protein database (PDB) archive (PDB ID: 4DAJ for M_3R; PDB ID: 3EK4 for cpGFP). **b**, Site-directed mutagenesis of residues in the N and C termini of the linker region. The numbers indicate amino acid positions in the linker region (the first on N-terminus as N1, and the first on C-terminus as C1). The candidate with the best response is shown in a black circle and is called ACh2.5, with the C4 residue mutated to K; this candidate is used for further engineering steps. **c**, Left: crystal structure of the cpEGFP moiety in the ACh3.0 sensor; targeted residues for mutagenesis screening are indicated in green and the corresponding amino acid labeled on the structure. Right, the fluorescence response of the indicated mutant candidate sensors is shown on top, with the sequences of the best-performing candidates on the bottom; the relative size of each letter reflects the probability of that amino acid in the sequence. The residues are named by the amino acid followed by the position in cpGFP (the first amino acid in cpGFP as N1). The crystal structures are from protein database (PDB) archive (PDB ID: 3EK4 for cpGFP). **d**, The fluorescence response of each candidate ACh sensor with combined mutations from the best-performing sites in the linker and cpEGFP. Each point is calculated from the average of >100 cells. **e**, left, illustration of the ligand binding pocket in M_3R, which was mutated from W to A. Right, fluorescence image of HEK293T cells expressing ACh3.0-mut. **f**, The fluorescence response of ACh3.0 and ACh3.0-mut to indicated concentration of ACh applied (n = 3 wells for each point, with each well averaging >100 cells). Scale bar represents 10 μm. All data are shown as mean value ± SEM, with the error bars or shaded regions indicating SEM.

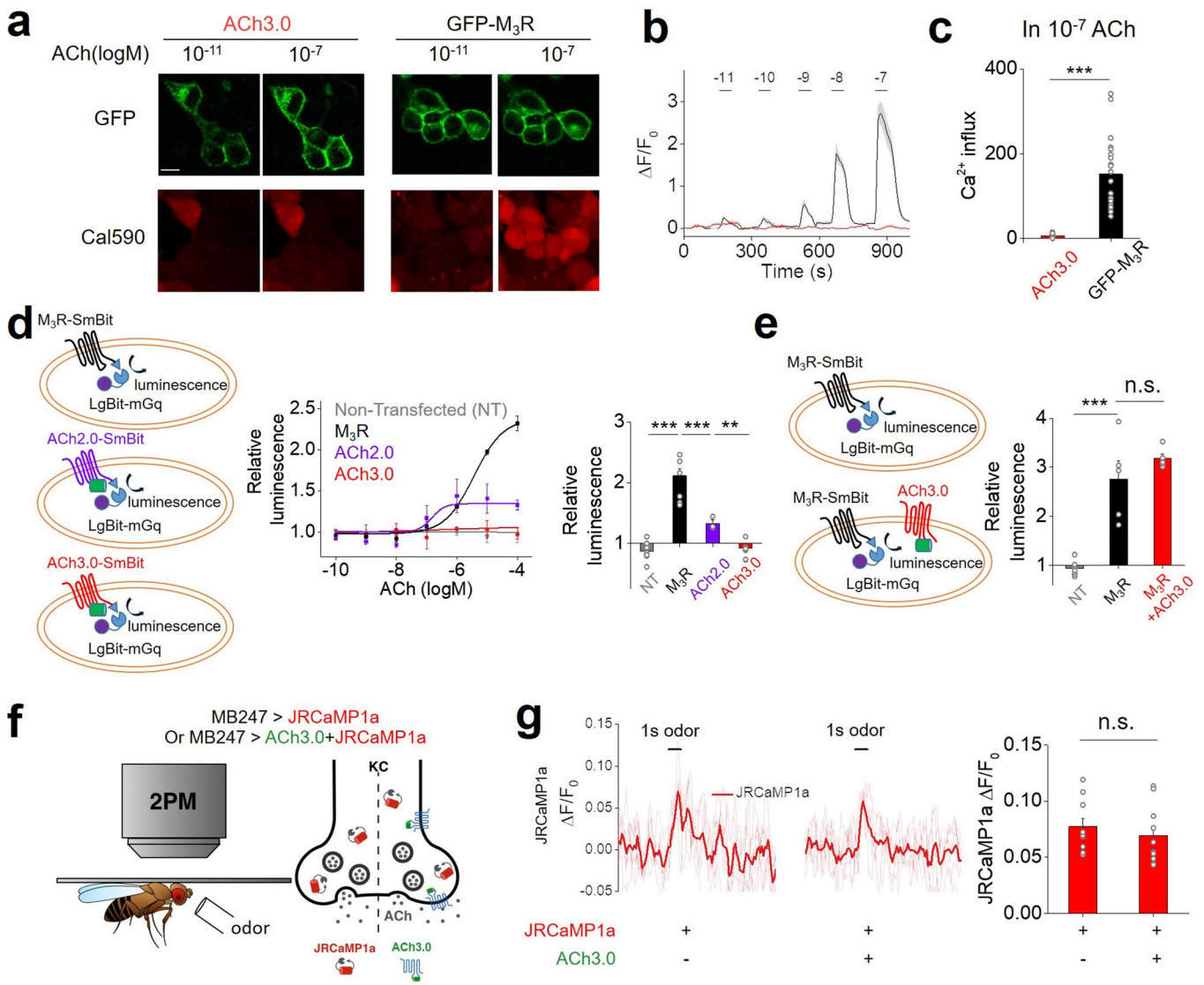


Extended Data Fig. 2 | Amino acid sequences of different cpGFP-based sensors. The summary of amino acids in linkers and critical residues within cpGFP in different genetically encoded sensors, including GPCR-based sensors and other protein backbone-based sensors.

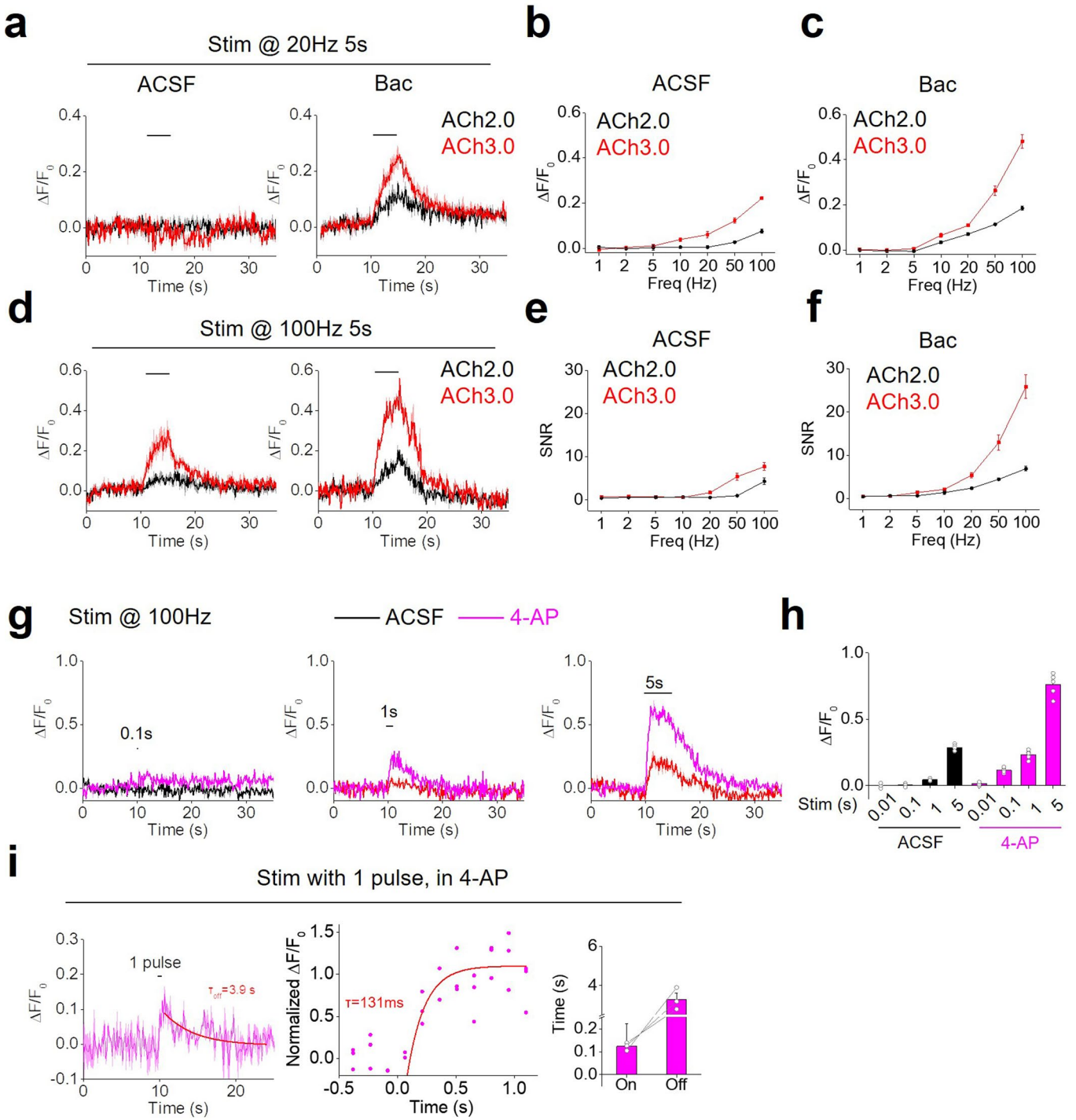


Extended Data Fig. 3 | See next page for caption.

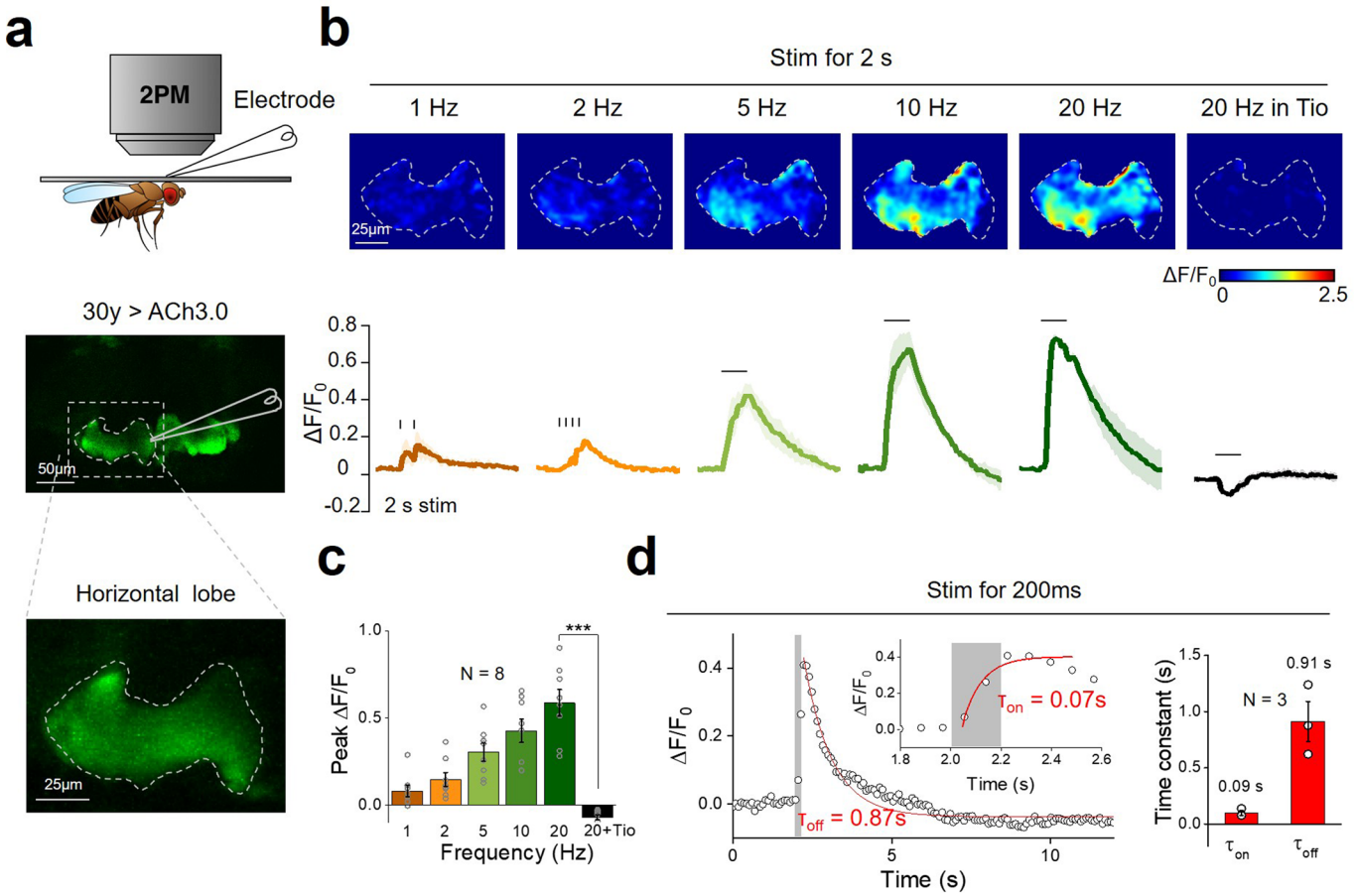
Extended Data Fig. 3 | Characterization of ACh2.0 and ACh3.0. a, The fluorescence response of ACh2.0, ACh3.0, and ACh3.0-mut to 100 μM ACh in HEK293T cells. The fluorescence images are shown on top, and corresponding pseudocolor images representing the signal-to-noise ratio (SNR) are shown at the bottom. Similar results as the representative images were observed for more than 7 cells. Scale bars represent 10 μm. **b**, The peak fluorescence response ($\Delta F/F_0$, left) and SNR (right) of ACh2.0 (black) and ACh3.0 (red) are measured with the indicated concentrations of ACh; n = 8 and 7 cells for ACh2.0 and ACh3.0, respectively. **c**, Example fluorescence images of ACh3.0 (left) and ACh3.0-mut (right) expressed in cultured rat cortical neurons. Membrane-targeted mScarlet-CAAX is co-expressed and used to confirm expression at the plasma membrane. Similar results as the representative images were observed for more than 5 neurons. Scale bars represent 10 μm in the original image and 5 μm in the magnified images. **d**, Representative traces (left) and group summary (right) of the fluorescence response of ACh2.0, ACh3.0, and ACh3.0-mut expressed in cultured neurons; where indicated, 100 μM ACh is applied to the cells (n = 4, 5, and 7 neurons for ACh2.0, ACh3.0, and ACh3.0-mut, respectively), p=9.45E-5 between ACh2.0 and ACh3.0; p=6.42E-5 between ACh3.0 and ACh3.0-mut. **e**, Left, representative traces of the normalized fluorescence change in ACh3.0 (red) and ACh3.0-mut (gray) in response to application of the indicated concentrations of ACh. Note that the ACh-induced fluorescence response in ACh3.0 is blocked by the M_3R antagonist tiotropium (Tio, 3 μM). Right, representative trace of the normalized fluorescence change in ACh3.0 in response to indicated compounds. ACh: 100 μM; nicotine (Nic): 50 μM; 5-HT: 1 μM; norepinephrine (NE): 10 μM; dopamine (DA): 20 μM; glutamate (Glu): 10 μM; and Tio: 2 μM. Similar results as the representative images were observed for more than 5 neurons. **f**, The excitation and emission spectra of ACh3.0 sensor in the absence (light green) and presence of ACh (100 μM, dark green). **g**, Left, pseudocolor images showing the fluorescence response of ACh3.0 in confocal line scanning mode, with indicated concentrations of ACh applied by bath application. Middle, exemplar fluorescence response trace of ACh3.0 to different concentrations of ACh applied. Right, group data of the ACh3.0 dose-dependent fluorescence response in line scanning mode (from n = 4 coverslips), which is used to estimate the local ACh concentration reaching the cells during kinetics experiments. The steady-state fluorescence response of ACh3.0 to puffed ACh are shown and calibrated based on the curve, with the detail numbers of 10 μM pipette ACh list as an example (pipette short as pip.; Estimated short as Esti.) All data are shown as mean value ± SEM, with the error bars or shaded regions indicating SEM. Two-sides Student's t test performed in (d); ***p<0.001.



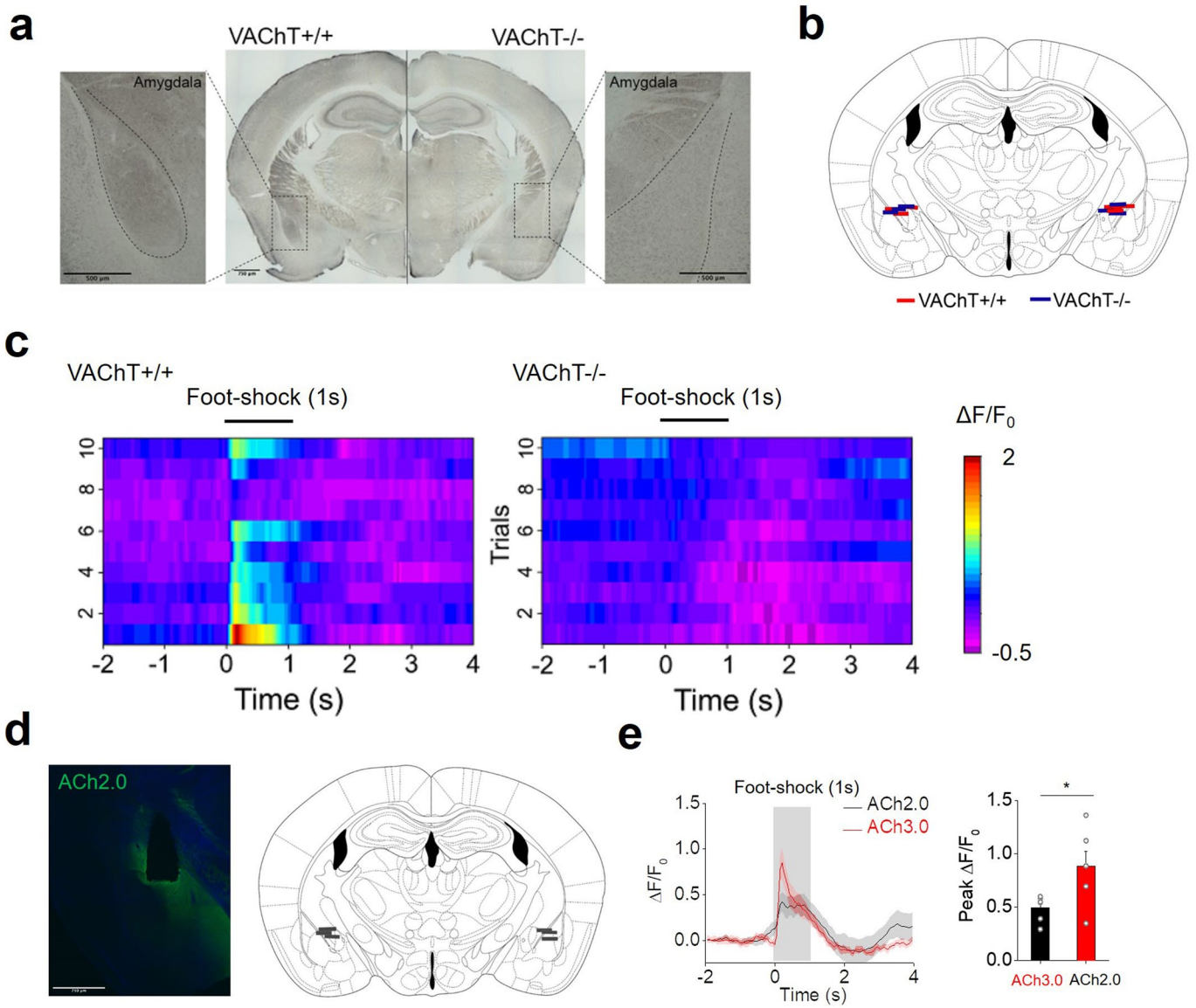
Extended Data Fig. 4 | The GRAB$_{ACh3.0}$ sensor produces negligible downstream signaling. a–c, HEK293T cells expressing either a GFP-tagged M_3R construct or ACh3.0 are loaded with the red Ca^{2+} dye Cal590 (a), and the change in Cal590 fluorescence is measured in response to various concentrations of ACh (b). The Ca^{2+} influx is calculated as the integration of Cal590 fluorescent signal ($\Delta F/F_0$) to ACh application. The group summary data for Ca^{2+} influx measured in response to 0.1 μM ACh are shown in panel c; n = 21 and 15 cells for GFP-M_3R and ACh3.0, respectively, p=1.06E-7. **d**, Left, cartoon illustrating the experimental design of the luciferase complementation assay, in which cells expressed M_3R-SmBit or ACh2.0/3.0-SmBit together with LgBit-mGq. Middle, the luminescence signal measured in non-transfected HEK293T cells (NT), cells expressing ACh2.0/ACh3.0-SmBit, or cells expressing M_3R-SmBit in response to application of the indicated concentrations of ACh, normalized to the signal measured in control buffer-treated cells (n = 6 wells for NT; n = 6 wells for M_3R; n = 3 wells for ACh2.0; n = 6 wells for ACh3.0, with >100 cells in each well). Right, group summary of the luminescence signal measured in response to 100 μM ACh (n = 6 wells for NT; n = 6 wells for M_3R; n = 3 wells for ACh2.0; n = 6 wells for ACh3.0, with >100 cells in each well; p=7.11E-7 between NT and M_3R; p=9.95E-7 between M_3R and ACh2.0; p=0.003 between ACh2.0 and ACh3.0). **e**, Similar to (d), except the luminescence signal is measured in HEK293T cells expressing M_3R-SmBit or cells expressing both M_3R-SmBit and ACh3.0. The group summary at the right shows the luminescence signal in response to 100 μM ACh; n = 5-8 wells per group, with each group averaging >100 cells, p=7.95E-5 between NT and M_3R; p=0.33 between M_3R and M_3R+ACh3.0. **f**, Schematic cartoon depicting two-photon imaging of transgenic flies in response to odorant stimulation. Ca^{2+} influx is measured by expressing jRCaMP1a either alone or together with ACh3.0 in the Kenyon cells (KC) of the mushroom body. **g**, Representative fluorescence traces (left) and group summary (right) of jRCaMP1a fluorescence measured in response to odorant application in flies expressing jRCaMP1a either alone or together with ACh3.0; n = 10 flies per group, p=0.49. All data are shown as mean value ± SEM, with the error bars or shaded regions indicating SEM. Scale bar represents 10 μm. Two-sides Student's t test performed in (c), (d), (e) and (g); ***p<0.001 and n.s., not significant.



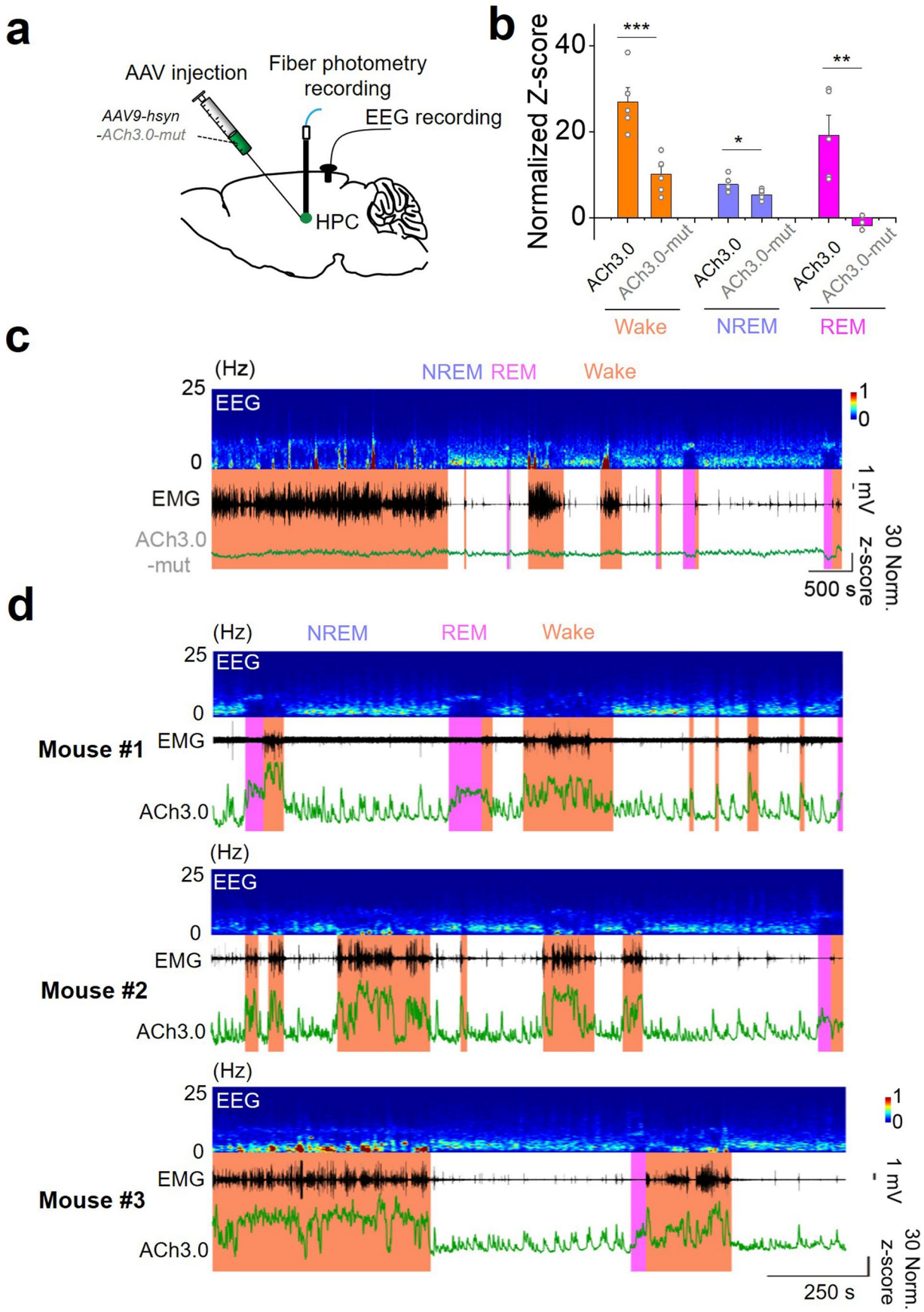
Extended Data Fig. 5 | Probing endogenous ACh release in mouse brain slices. a–f, Representative fluorescence traces (a, d) and group summary (b, c, e, f) of the fluorescence change ($\Delta F/F_0$ and SNR) in neurons expressing either ACh2.0 or ACh3.0 in response to electrical stimulation in MHb-IPN brain slices. The slices are bathed in either ACSF or 2 μM baclofen (Bac). N=5 slices from 3 mice for ACh2.0, and n = 10 slices from 7 mice for ACh3.0. **g**, **h**, The representative fluorescence traces (g) and group data (h) of ACh3.0-expressing neurons in response to 100-Hz electrical stimulation with different stimulation times in MHb-IPN brain slices. The response in either ACSF or 100 μM 4-AP is measured and summarized; n = 5 slices from 5 mice. **i**, The kinetics of fluorescence response of ACh3.0 to a single pulse (2 ms) of electrical stimulation in the presence of 100 μM 4-AP. The response in three independent experiments are normalized and plotted together in the middle. The group data of on and off response time constants are summarized on the right (n = 3 slices from 3 mice). All data are shown as mean value ± SEM, with the error bars or shaded regions indicating SEM.



Extended Data Fig. 6 | Monitoring *in vivo* ACh release induced by electrical stimulation in *Drosophila*. a, Schematic illustration depicting the experiment in which a transgenic fly expressing ACh3.0 in the KC cells in the mushroom body is placed under a two-photon microscope, and a glass electrode is placed near the mushroom body and used to deliver electrical stimuli. The fly brain is bathed in AHLS containing 100 μM nicotinic acetylcholine receptor blocker mecamylamine (Meca). **b**, Pseudocolor images (top) and representative traces (bottom) of the fluorescence change in ACh3.0 in response to 2 s of electrical stimulation at the indicated frequencies. Where indicated, the M_3R antagonist tiotropium (Tio, 10 μM) is applied to the bath solution. Similar results as the representative images were observed for 8 flies. **c**, Group summary of the data shown in panel (b); n = 8 flies, p=0.0004. **d**, ACh3.0 fluorescence is measured before and after a 200-ms electrical stimulation, and the rise and decay phases are fitted with a single-exponential function; the time constants are indicated and summarized on the right; n = 3 flies. All data are shown as mean value ± SEM, with the error bars or shaded regions indicating SEM. Two-sides Student's t test performed in (c); ***p<0.001.

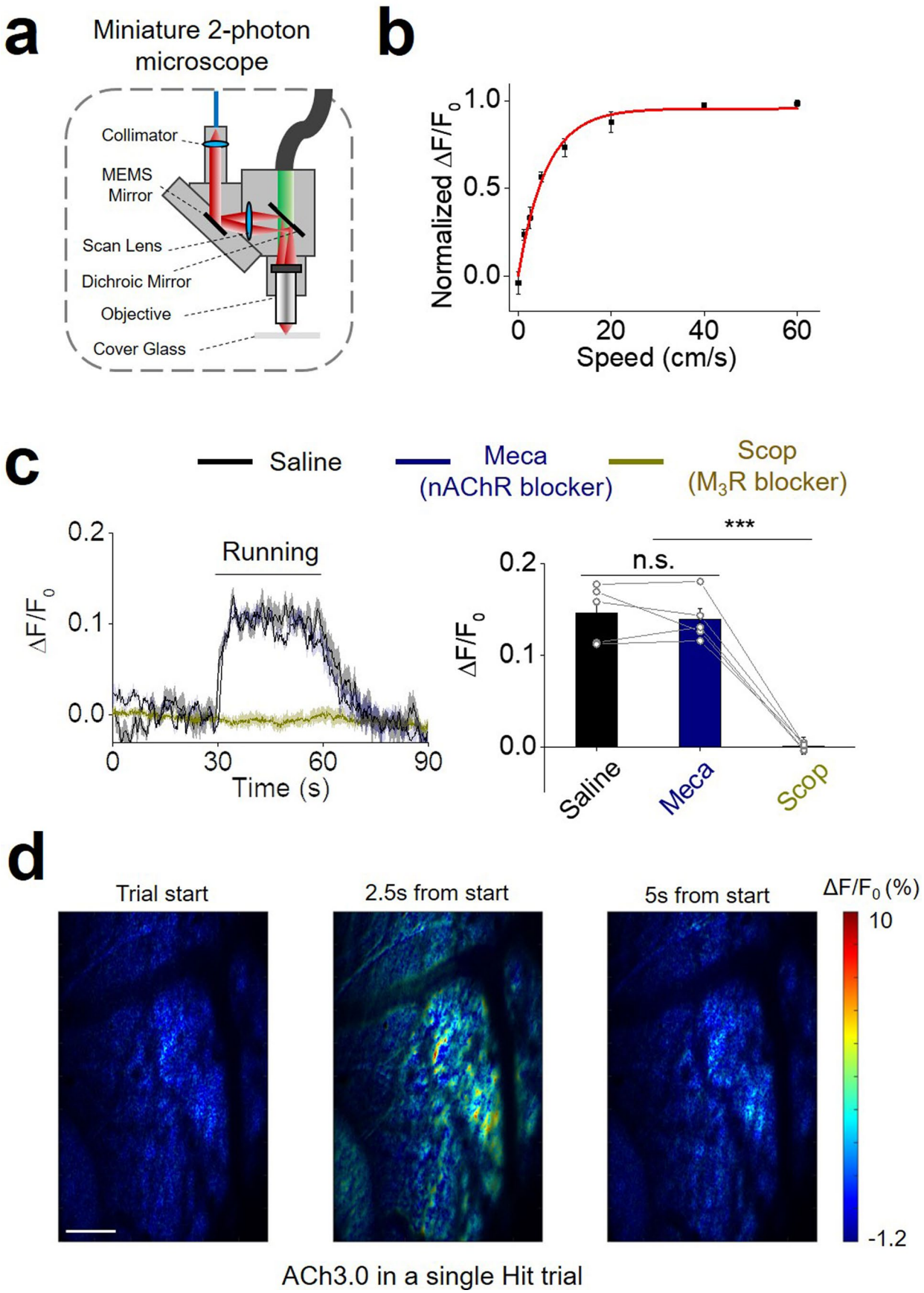


Extended Data Fig. 7 | Monitoring endogenous cholinergic signals in BLA of mice *in vivo*. a, VAChT immunohistochemistry is performed in coronal mouse sections obtained from a control ($VAChT^{+/+}$) mouse (left) and a $VAChT^{-/-}$ mouse (right). The insets show magnified views of cholinergic terminals in the basolateral amygdala (BLA). Similar results as the representative images were observed for 6 mice. Scale bars represent 500 μm. **b**, Diagram summarizing the location of the optic fiber terminals in the BLA of $VAChT^{+/+}$ (red) and $VAChT^{-/-}$ mice (blue); n = 6 mice per group. This image is modified from the brain map in Allen mouse brain atlas (Allen Mouse Brain Atlas (2004), Allen Institute for Brain Science). **c**, Pseudocolor image showing the change in ACh3.0 fluorescence measured in the BLA of $VAChT^{+/+}$ mice (left) and $VAChT^{-/-}$ mice (right) in response to a 1-s foot-shock; ten consecutive trials are shown. **d**, **e**, The comparison of ACh2.0 and ACh3.0 fluorescence response in BLA of mice to foot-shock stimuli. The fluorescence signal showing the expression of ACh2.0 and the fiber photometry recording sites are shown in (d). Fluorescence traces and group data of the 1 s foot-shock induced fluorescence response in ACh2.0 (black) and ACh3.0 (red) are shown in (e) (n = 6 mice each for ACh3.0 and ACh2.0, p=0.04). Scale bar: 750 μm. All data are shown as mean value ± SEM, with the error bars or shaded regions indicating SEM. Two-sides Student's t test performed in (e); *, p<0.05.



Extended Data Fig. 8 | See next page for caption.

Extended Data Fig. 8 | Recording of ACh signal during sleep-wake cycle. a, The schematic illustration and representative recording data of ACh3.0-mut sensor during the sleep-wake cycle in mice. **b**, The group data of the fluorescence response of ACh3.0 and ACh3.0-mut sensors in different sleep-wake status (n = 5 mice for ACh3.0 and n = 6 mice for ACh3.0-mut, p=0.0009 in wake; p=0.049 in NREM; p=0.008 in REM). **c**, The representative recording data of ACh3.0-mut sensor during the sleep-wake cycle in mice. Similar results as the representative images were observed for 6 mice. **d**, Multiple recording traces of the ACh3.0 sensor during the sleep-wake cycle (from 3 mice, additional to the representative one in Fig. 3g). All data are shown as mean value ± SEM, with the error bars or shaded regions indicating SEM. Two-sides Student's t test performed in (b); *, p<0.05; **, p<0.01; ***, p<0.001.



Extended Data Fig. 9 | See next page for caption.

Extended Data Fig. 9 | Imaging of ACh signal in the cortex. a, Cartoon illustration of the miniature two-photon microscope. **b**, Group data of ACh3.0 fluorescence in mice recorded while running on a treadmill at indicated speeds (n = 5 mice). **c**, Representative traces and group summary of ACh3.0 fluorescence measured in mice while performing the running task; where indicated, the mice receive an i.p. injection of saline (black), the nAChR blocker mecamylamine (Meca, 2 mg/kg body weight, blue), or the M_3R antagonist scopolamine (Scop, 20 mg/kg body weight, dark yellow); each trace is averaged from 10 trials; n = 5 mice per group, p=0.54 between Saline and Meca; p=0.0002 between Meca and Scop. **d**, Pseudocolor images showing the ACh3.0 fluorescence response in the S1 in the Hit trial of the whisker-guided object location discrimination task. The left, middle and right image showing the response during baseline, peak in the answer period and after response. Similar results as the representative images were observed for 3 mice. Scale bar: 100 μm. All data are shown as mean value ± SEM, with the error bars or shaded regions indicating SEM. Two-sides Student's t test performed in (c); *p<0.05, **p<0.01, ***p<0.001, and n.s., not significant.