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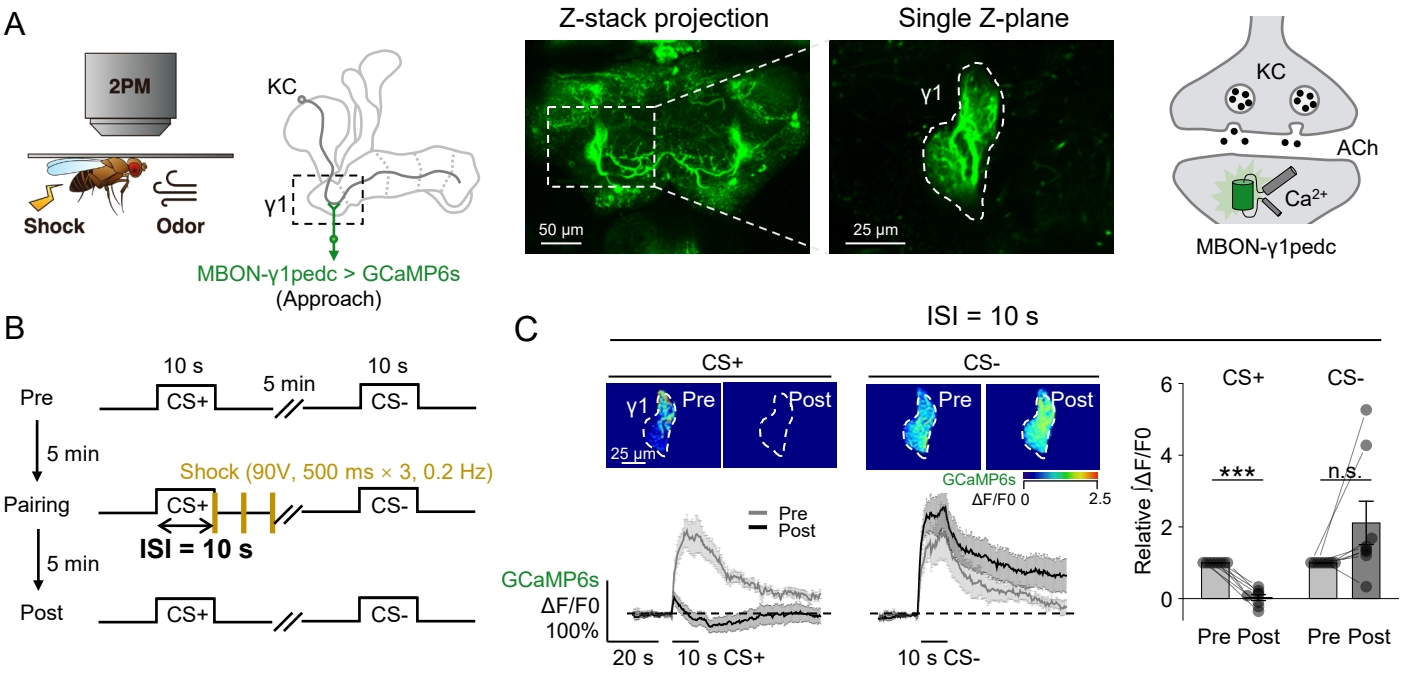
Supplemental information

Local 5-HT signaling bi-directionally regulates the coincidence time window for associative learning

Jianzhi Zeng, Xuelin Li, Renzimo Zhang, Mingyue Lv, Yipan Wang, Ke Tan, Xiju Xia, Jinxia Wan, Miao Jing, Xiuning Zhang, Yu Li, Yang Yang, Liang Wang, Jun Chu, Yan Li, and Yulong Li

Figure S1


A
2PM
Shock
Odor
KC
γ1
MBON-γ1pedc > GCaMP6s
(Approach)
Z-stack projection
Single Z-plane
50 μm
25 μm
KC
ACh
Ca2+
MBON-γ1pedc
B
Pre
10 s
CS+
5 min
10 s
CS-
5 min
Shock (90V, 500 ms × 3, 0.2 Hz)
Pairing
ISI = 10 s
5 min
Post
C
ISI = 10 s
CS+
CS-
Pre
Post
GCaMP6s
ΔF/F0 0
2.5
GCaMP6s
ΔF/F0
100%
20 s
10 s CS+
10 s CS-
Relative ∫ΔF/F0
***
n.s.
Pre Post
Pre Post

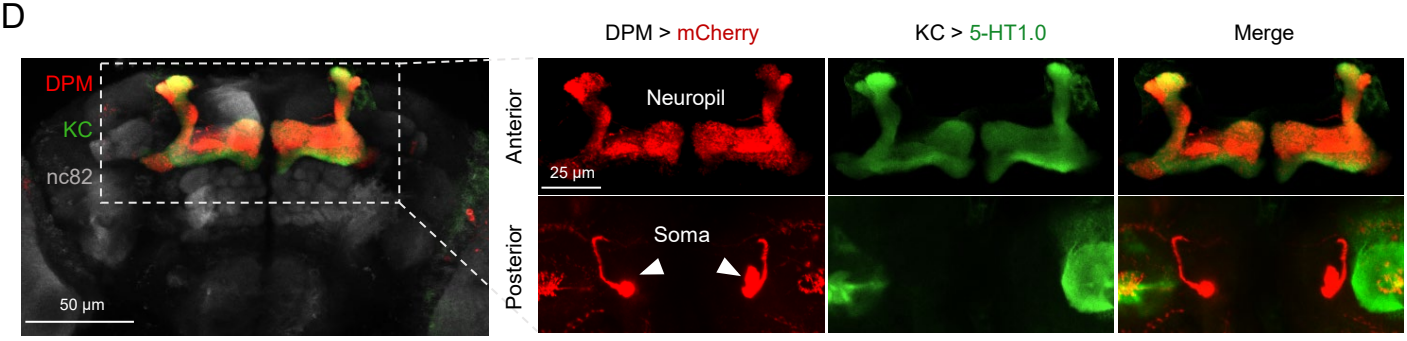

D
DPM
KC
nc82
50 μm
DPM > mCherry
KC > 5-HT1.0
Merge
Anterior
Posterior
Neuropil
Soma
25 μm

Figure S1. Ca^{2+} imaging in MBON-γ1pedc and the anatomy of the DPM neuron, related to Figures 2 and 3.

(**A-C**) The Ca^{2+} signal within MBON-γ1pedc reveals synaptic depression after odor-shock pairing. Schematics depicting the *in vivo* two-photon imaging setup, representative fluorescence images (**A**), and the experimental protocol (**B**) for measuring changes of the Ca^{2+} signal with GCaMP6s expressed in the MBON-γ1pedc. Representative pseudo-color images (**C, top left**), average (±SEM) traces (**C, bottom left**) and group analysis (**C, right**) of the fluorescence signals of GCaMP6s in the pre- and post-pairing sessions; n=8 flies/group.

(**D**) Immunofluorescent images (Z-stack projection) of a dissected brain from the fly expressing CsChrimson-mCherry (red) in the DPM neuron and 5-HT1.0 (green) in the KCs. The brain was counterstained with the anti-nc82 (gray). The somata of two DPM neurons in the posterior side and the neuropil in the anterior side were indicated.

***p<0.001; and n.s., not significant (paired Student's t-test).

Figure S2

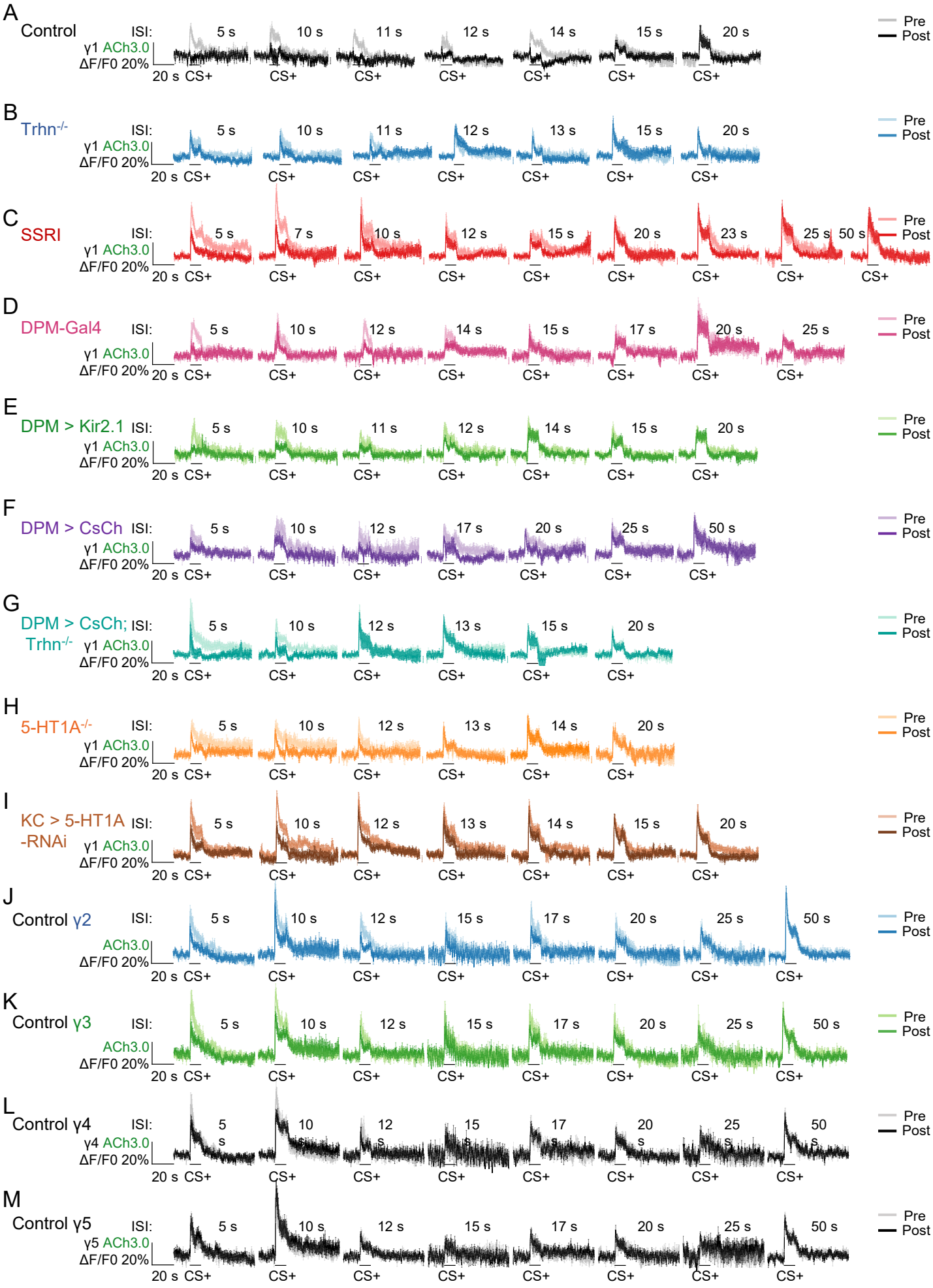

A
Control
ISI:
γ1 ACh3.0
ΔF/F0 20%
5 s
10 s
11 s
12 s
14 s
15 s
20 s
20 s CS+
CS+
Pre
Post
B
Trhn-/-
ISI:
γ1 ACh3.0
ΔF/F0 20%
5 s
10 s
11 s
12 s
13 s
15 s
20 s
20 s CS+
CS+
Pre
Post
C
SSRI
ISI:
γ1 ACh3.0
ΔF/F0 20%
5 s
7 s
10 s
12 s
15 s
20 s
23 s
25 s
50 s
20 s CS+
CS+
Pre
Post
D
DPM-Gal4
ISI:
γ1 ACh3.0
ΔF/F0 20%
5 s
10 s
12 s
14 s
15 s
17 s
20 s
25 s
20 s CS+
CS+
Pre
Post
E
DPM > Kir2.1
ISI:
γ1 ACh3.0
ΔF/F0 20%
5 s
10 s
11 s
12 s
14 s
15 s
20 s
20 s CS+
CS+
Pre
Post
F
DPM > CsCh
ISI:
γ1 ACh3.0
ΔF/F0 20%
5 s
10 s
12 s
17 s
20 s
25 s
50 s
20 s CS+
CS+
Pre
Post
G
DPM > CsCh;
Trhn-/-
ISI:
γ1 ACh3.0
ΔF/F0 20%
5 s
10 s
12 s
13 s
15 s
20 s
20 s CS+
CS+
Pre
Post
H
5-HT1A-/-
ISI:
γ1 ACh3.0
ΔF/F0 20%
5 s
10 s
12 s
13 s
14 s
20 s
20 s CS+
CS+
Pre
Post
I
KC > 5-HT1A
-RNAi
ISI:
γ1 ACh3.0
ΔF/F0 20%
5 s
10 s
12 s
13 s
14 s
15 s
20 s
20 s CS+
CS+
Pre
Post
J
Control γ2
ISI:
ACh3.0
ΔF/F0 20%
5 s
10 s
12 s
15 s
17 s
20 s
25 s
50 s
20 s CS+
CS+
Pre
Post
K
Control γ3
ISI:
ACh3.0
ΔF/F0 20%
5 s
10 s
12 s
15 s
17 s
20 s
25 s
50 s
20 s CS+
CS+
Pre
Post
L
Control γ4
ISI:
γ4 ACh3.0
ΔF/F0 20%
5 s
10 s
12 s
15 s
17 s
20 s
25 s
50 s
20 s CS+
CS+
Pre
Post
M
Control γ5
ISI:
γ5 ACh3.0
ΔF/F0 20%
5 s
10 s
12 s
15 s
17 s
20 s
25 s
50 s
20 s CS+
CS+
Pre
Post

Figure S2. Changes in synaptic plasticity of ACh release from different γ lobe compartments of control flies, and flies with various genetic or pharmacological manipulations, related to Figures 2, 5 and 6.

Shown are average (±SEM) traces of the odor-evoked change in ACh3.0 fluorescence measured in pre- and post-pairing sessions with the indicated ISI.

Figure S3

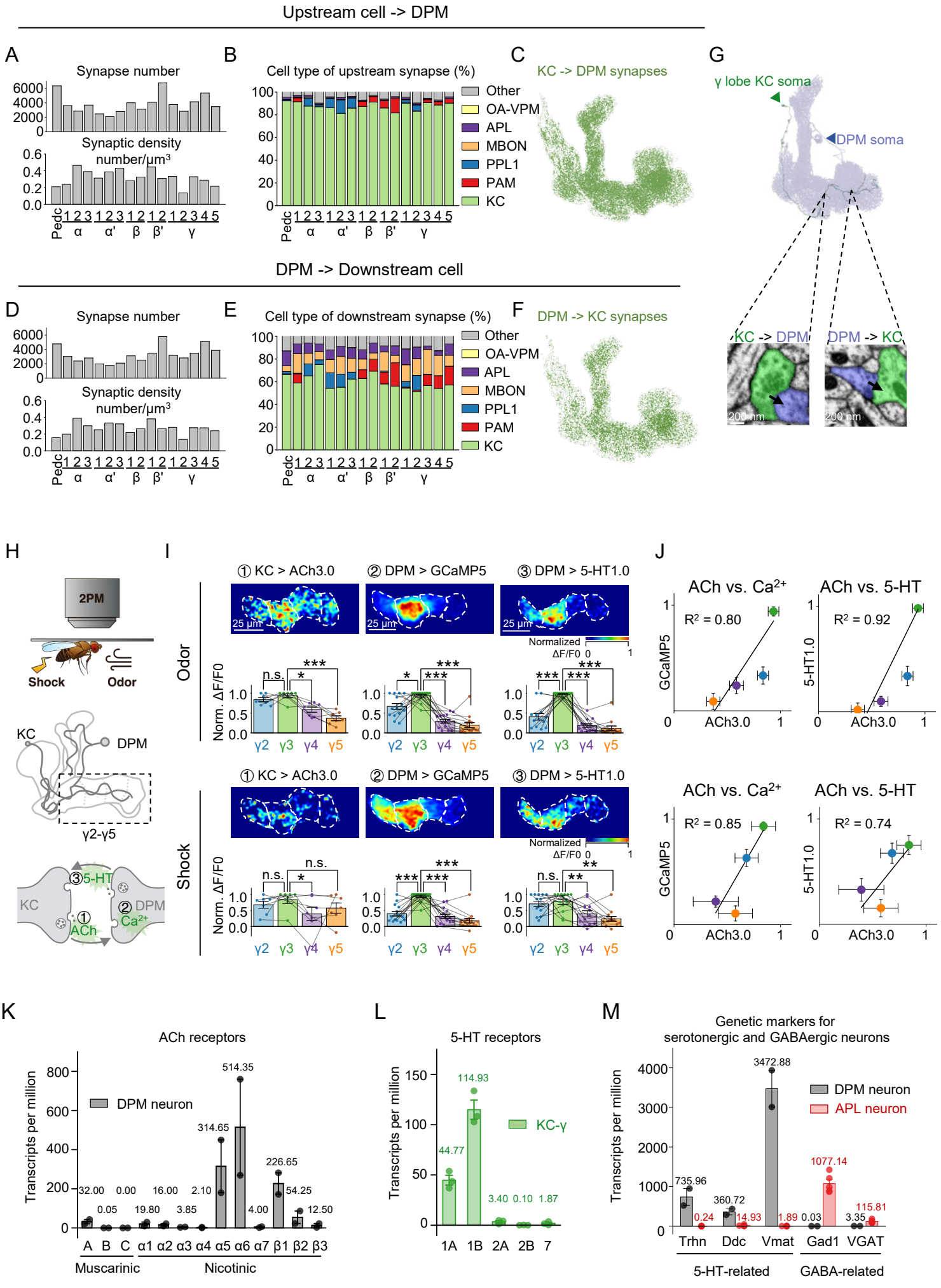

Upstream cell -> DPM
A
Synapse number
Synaptic density number/μm3
B
Cell type of upstream synapse (%)
Other
OA-VPM
APL
MBON
PPL1
PAM
KC
C
KC -> DPM synapses
G
γ lobe KC soma
DPM soma
KC -> DPM
DPM -> KC
200 nm
DPM -> Downstream cell
D
E
Cell type of downstream synapse (%)
F
DPM -> KC synapses
H
2PM
Shock
Odor
KC
DPM
γ2-γ5
③5-HT
② DPM Ca2+
① ACh
I
① KC > ACh3.0
② DPM > GCaMP5
③ DPM > 5-HT1.0
Odor
Shock
25 μm
Normalized ΔF/F0
Norm. ΔF/F0
n.s.
γ2 γ3 γ4 γ5
J
ACh vs. Ca2+
ACh vs. 5-HT
R2 = 0.80
R2 = 0.92
R2 = 0.85
R2 = 0.74
GCaMP5
5-HT1.0
ACh3.0
K
ACh receptors
Transcripts per million
DPM neuron
32.00 0.00 0.05 16.00 19.80 2.10 3.85 314.65 514.35 4.00 226.65 54.25 12.50
A B C α1 α2 α3 α4 α5 α6 α7 β1 β2 β3
Muscarinic
Nicotinic
L
5-HT receptors
KC-γ
44.77 114.93 3.40 0.10 1.87
1A 1B 2A 2B 7
M
Genetic markers for serotonergic and GABAergic neurons
DPM neuron
APL neuron
735.96 0.24 360.72 14.93 3472.88 1.89 0.03 1077.14 3.35 115.81
Trhn Ddc Vmat Gad1 VGAT
5-HT-related
GABA-related

Figure S3. The DPM neuron and KCs are reciprocally connected and functionally correlated, related to Figures 3, 4 and 7.

(**A-G**) Analyzing MB EM connectomics reveals reciprocal connections between the DPM neuron and KCs. Shown are summary of the number of synapses (**A** and **D, top**), synaptic density (**A** and **D, bottom**), the percentage of synapses from indicated cell types (**B** and **E**), and the reconstruction of synapses from KCs (**C** and **F**) that are upstream or downstream of the DPM neuron in MB compartments. Also shown are reconstruction (**G**, **top**) and representative EM images (**G**, **bottom**) of a KC (green) forming reciprocal connections with the DPM neuron (blue) in the γ lobe. The arrows in the EM images indicate the direction from the presynapse to the postsynapse. The arrow heads in the reconstruction indicate the somata. Pedc, peduncle; OA-VPM, octopaminergic ventral paired median neuron; APL, anterior paired lateral neuron; MBON, mushroom body output neuron; PPL1, paired posterior lateral 1 cluster neuron; PAM, protocerebral anterior medial cluster neuron; KC, Kenyon cell. Version 1.1 of the hemibrain connectome [S1] was used for the analysis, and synapses with a confidence value >0.75 were included.

(**H-J**) The heterogenous patterns of 5-HT and Ca^{2+} dynamics of the DPM neuron are directly correlated with ACh release from KCs. Schematics (**H**), normalized pseudocolor images and summary (**I**) of ACh, Ca^{2+} and 5-HT signals in response to odor (1 s) or electric shock (0.5 s, 90 V) in the γ2-γ5 compartments; n=7-14 flies/group. For each fly, the fluorescence signals were normalized to the compartment with the highest response. Correlation analyses were performed by plotting the changes in Ca^{2+} or 5-HT dynamics (*y*-axis) against the changes in ACh release (*x*-axis) (**J**). The data were fitted to a linear function, and the R^2 is shown.

(**K-M**) Transcriptomic analysis of the DPM neuron, KCs of the γ lobe, and the APL neuron. The mAChRs and nAChRs in the DPM neuron (**K**; each point contains 123-130 cells). The 5-HT receptors in KCs of the γ lobe (**L**; each point contains ~2500 cells). The genetic markers for serotonergic and GABAergic neuron in the DPM neuron and the APL neuron (**M**; each point contains 123-130 DPM neurons or 20-36 APL neurons). Trhn, tryptophan hydroxylase neuronal; Ddc, DOPA-decarboxylase; Vmat, vesicular monoamine transporter; Gad1, glutamic acid decarboxylase 1; VGAT, vesicular GABA transporter. The published transcript database [S2] was used for analysis.

**p*<0.05; ***p*<0.01; ****p*<0.001; and n.s., not significant (paired Student's *t*-test).

Figure S4

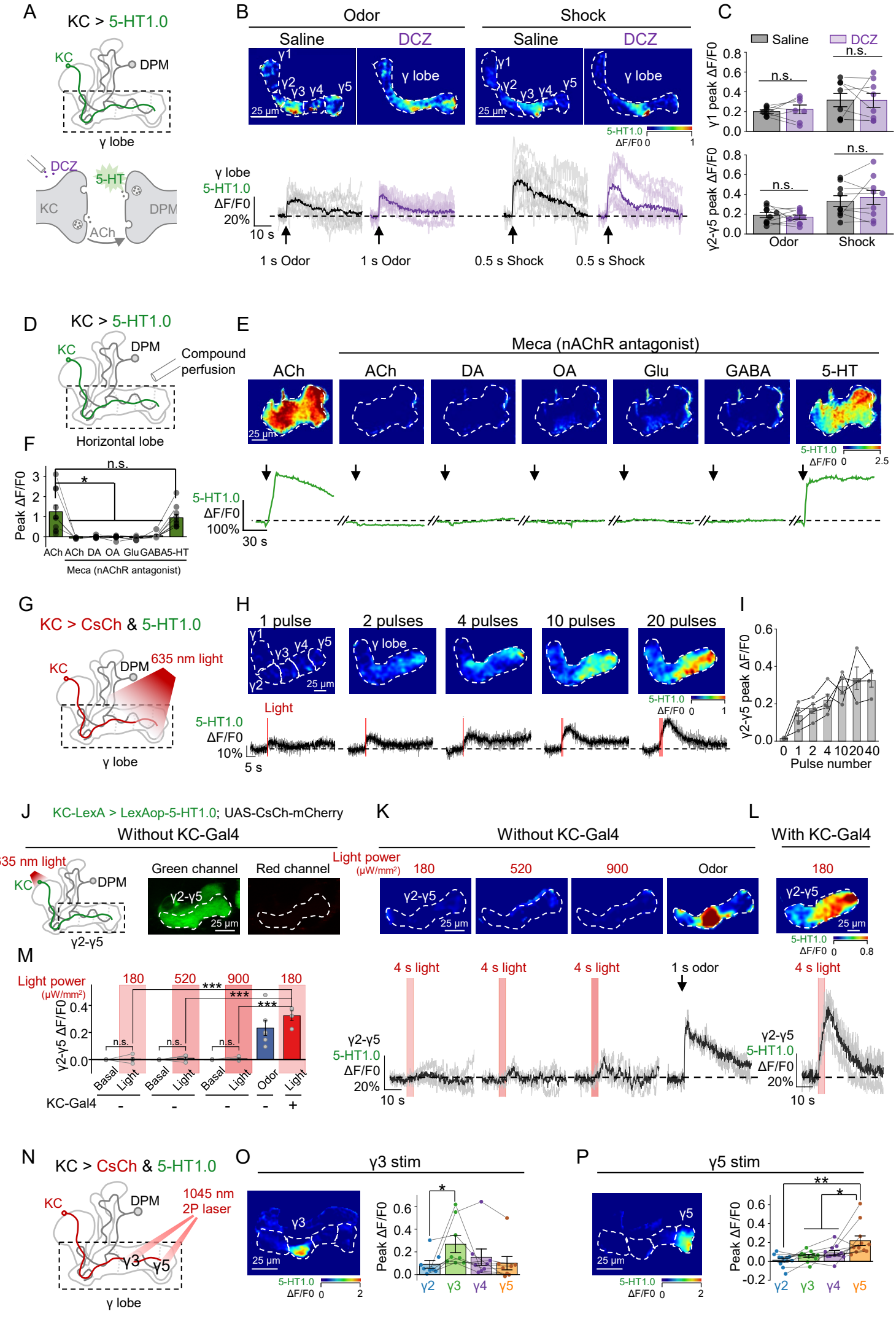

A
KC > 5-HT1.0
KC
DPM
γ lobe
DCZ
5-HT
ACh
B
Odor
Shock
Saline
DCZ
γ1
γ2
γ3 γ4
γ5
γ lobe
25 μm
5-HT1.0
ΔF/F0 0
1
γ lobe
5-HT1.0
ΔF/F0
20%
10 s
1 s Odor
1 s Odor
0.5 s Shock
0.5 s Shock
C
Saline
DCZ
n.s.
γ1 peak ΔF/F0
γ2-γ5 peak ΔF/F0
Odor
Shock
D
KC > 5-HT1.0
KC
DPM
Compound perfusion
Horizontal lobe
E
Meca (nAChR antagonist)
ACh
ACh
DA
OA
Glu
GABA
5-HT
25 μm
5-HT1.0
ΔF/F0 0
2.5
5-HT1.0
ΔF/F0
100%
30 s
F
n.s.
*
Peak ΔF/F0
ACh ACh DA OA Glu GABA 5-HT
Meca (nAChR antagonist)
G
KC > CsCh & 5-HT1.0
KC
DPM
635 nm light
γ lobe
H
1 pulse
2 pulses
4 pulses
10 pulses
20 pulses
γ1
γ2
γ3 γ4
γ5
γ lobe
25 μm
5-HT1.0
ΔF/F0 0
1
Light
5-HT1.0
ΔF/F0
10%
5 s
I
γ2-γ5 peak ΔF/F0
0 1 2 4 10 20 40
Pulse number
J
KC-LexA > LexAop-5-HT1.0; UAS-CsCh-mCherry
Without KC-Gal4
635 nm light
KC
DPM
γ2-γ5
Green channel
Red channel
γ2-γ5
25 μm
K
Without KC-Gal4
Light power
(μW/mm²)
180
520
900
Odor
γ2-γ5
25 μm
L
With KC-Gal4
180
γ2-γ5
25 μm
5-HT1.0
ΔF/F0 0
0.8
M
Light power
(μW/mm²)
180
520
900
180
***
***
***
n.s.
n.s.
n.s.
γ2-γ5 ΔF/F0
Basal
Light
Basal
Light
Basal
Light
Odor
Light
KC-Gal4
- - - - +
4 s light
4 s light
4 s light
1 s odor
4 s light
γ2-γ5
5-HT1.0
ΔF/F0
20%
10 s
γ2-γ5
5-HT1.0
ΔF/F0
20%
10 s
N
KC > CsCh & 5-HT1.0
KC
DPM
1045 nm
2P laser
γ3
γ5
γ lobe
O
γ3 stim
γ3
25 μm
5-HT1.0
ΔF/F0 0
2
*
Peak ΔF/F0
γ2 γ3 γ4 γ5
P
γ5 stim
γ5
25 μm
5-HT1.0
ΔF/F0 0
2
**
*
Peak ΔF/F0
γ2 γ3 γ4 γ5

Figure S4. KCs release ACh to trigger 5-HT release from the DPM neuron, related to Figure 4.

(**A-C**) The hM4Di agonist DCZ does not cause significant effect on odor or shock-evoked 5-HT signals in the γ lobe. Shown are schematics depicting the *in vivo* imaging setup in which 5-HT was measured in the γ lobe using 5-HT1.0 expressed in KCs in the absence or presence of 30 nM DCZ (**A**). Also shown are representative pseudocolor images (**B**, **top**), average and individual traces (**B**, **bottom**), and group analysis of the change in 5-HT1.0 fluorescence from the γ1 compartment (**C, top**) and γ2-γ5 compartments (**C, bottom**); n=7-9 flies/group. In each fly, the experiment was divided into saline and DCZ sessions, and in each session the odor and/or shock stimuli were applied for 1-3 trials, in random order.

(**D-F**) ACh application induces the release of 5-HT via nAChRs. Shown are schematics depicting the *in vivo* imaging setup in which 5-HT was measured in the γ lobe using 5-HT1.0 expressed in KCs (**D**). Also shown are representative pseudocolor images (**E**, **top**), single-trial traces (**E**, **bottom**), and summary of the change in 5-HT1.0 fluorescence from the MB horizontal lobe that includes the γ lobe (**F**) in response to application of the indicated neurotransmitters (1 mM) in the absence or presence of the nAChR antagonist Meca (100 μM). ACh, acetylcholine; DA, dopamine; OA, octopamine; Glu, glutamate; GABA, gamma-aminobutyric acid.

(**G-I**) Optogenetically activating KCs induces a pulse number–dependent release of 5-HT in the γ lobe. Shown are schematics (**G**) depicting the *in vivo* imaging setup in which CsChrimson-expressing KCs were activated by light pulses (1 ms/pulse, 635 nm, 10 Hz), and 5-HT was measured in the γ lobe using 5-HT1.0 expressed in KCs. Also shown are representative pseudocolor images (**H, top**), average and individual traces (**H, bottom**), and summary of the change in 5-HT1.0 fluorescence in response to the indicated number of light pulses; n=4 flies. For each fly, experiments were divided into 3 sessions, and in each session 1, 2, 4, 10 and 20 pulses were delivered randomly.

(**J-M**) Light stimulation does not affect 5-HT dynamics in flies with UAS-CsChrimson but without KC-Gal4 driver, ruling out the unspecific effect caused by leaky expression of channelrhodopsin. Shown are schematics (**J, left**) and fluorescence images (**J, right**) depicting the *in vivo* imaging setup in which 5-HT was measured with 5-HT1.0 expressed in KCs, while the light pulses (1 ms/pulse, 635 nm, 10 Hz) were delivered to the brain of the fly only carrying UAS-CsCh-mCherry, but not KC-Gal4. Also shown are representative pseudocolor images (**K, L, top**), average and individual traces (**K, L, bottom**), and group analysis (**M**) of the change in 5-HT1.0 fluorescence in response to light pulses (4 s) with the indicated power or odor stimuli (1 s), in flies without or with KC-Gal4. Note that the data of flies with KC-Gal4 are reproduced from the saline group of Figures 4E-4F. Also note that light pulses with the power of 180 $\mu W/mm^2$ were used in Figure 4D-4F and S4G-S4I.

(**N-P**) Optogenetically activating KCs in a spatially restricted manner induces local release of 5-HT. Shown are schematics (**N**) depicting the *in vivo* imaging setup in which a two-photon laser (1045-nm, 100-ms duration) was used to locally activate CsChrimson-expressing KCs and the 5-HT was measured in 5-HT1.0-expressing KCs. Also shown are representative pseudocolor images (**O and P**, **left**) and group analysis (**O and P, right**) of the change in 5-HT1.0 fluorescence in response to localized optogenetic stimulation in the γ3 or γ5 compartment; n=8-11 flies/group.

**p*<0.05; ***p*<0.01; ****p*<0.001; and n.s., not significant (paired or unpaired Student's *t*-test).

Figure S5

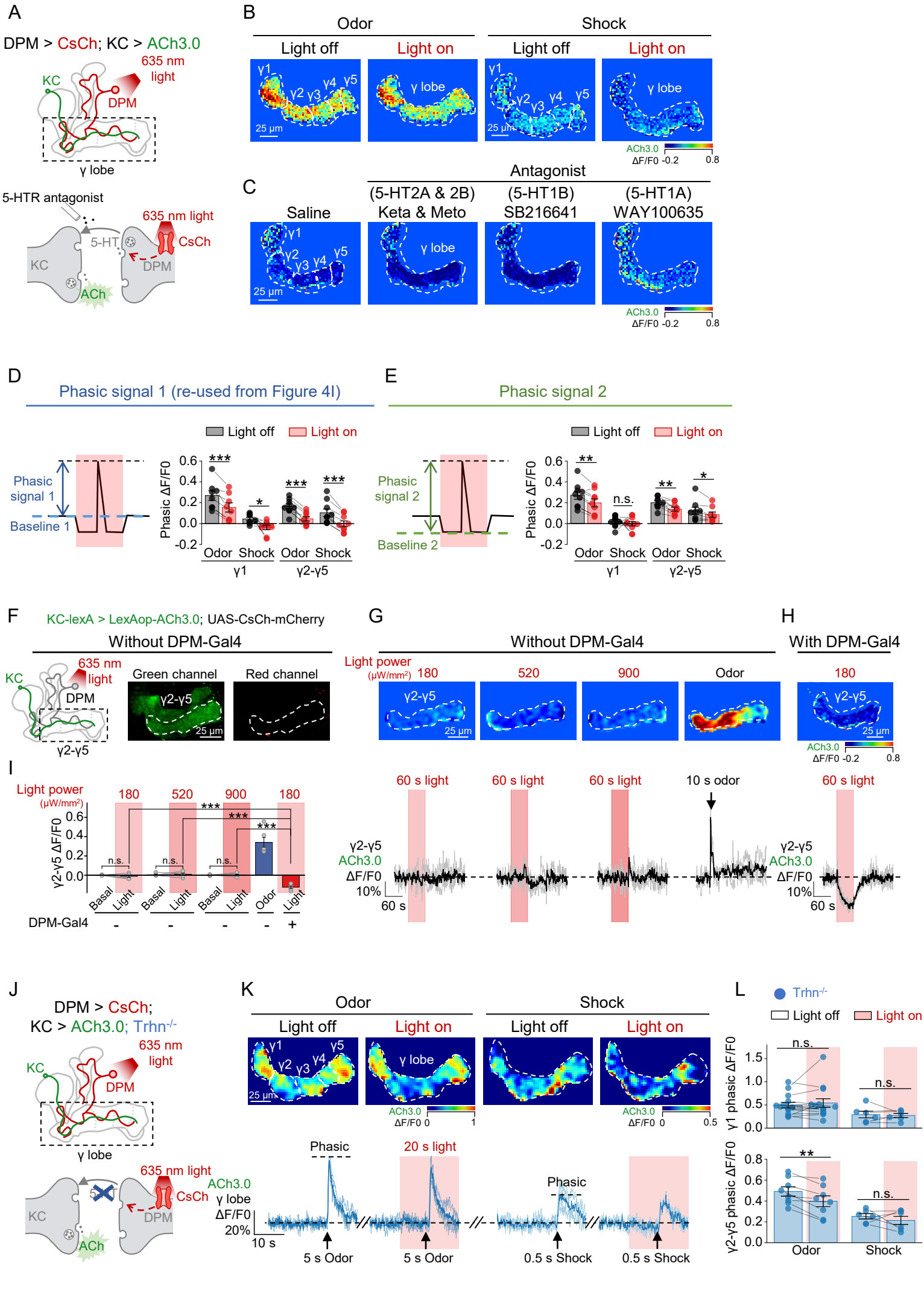

A
DPM > CsCh; KC > ACh3.0
635 nm light
KC
DPM
γ lobe
5-HTR antagonist
635 nm light
5-HT
CsCh
KC
DPM
ACh
B
Odor
Shock
Light off
Light on
Light off
Light on
γ1
γ2
γ3
γ4
γ5
γ lobe
25 μm
ACh3.0
ΔF/F0 -0.2
0.8
C
Antagonist
Saline
(5-HT2A & 2B)
Keta & Meto
(5-HT1B)
SB216641
(5-HT1A)
WAY100635
γ1
γ2
γ3
γ4
γ5
γ lobe
25 μm
ACh3.0
ΔF/F0 -0.2
0.8
D
Phasic signal 1 (re-used from Figure 4I)
Light off
Light on
Phasic signal 1
Baseline 1
Phasic ΔF/F0
0.6
0.4
0.2
0.0
-0.2
***
*
***
***
Odor
Shock
Odor
Shock
γ1
γ2-γ5
E
Phasic signal 2
Light off
Light on
Phasic signal 2
Baseline 2
Phasic ΔF/F0
0.6
0.4
0.2
0.0
-0.2
**
n.s.
**
*
Odor
Shock
Odor
Shock
γ1
γ2-γ5
F
KC-lexA > LexAop-ACh3.0; UAS-CsCh-mCherry
Without DPM-Gal4
635 nm light
KC
DPM
γ2-γ5
Green channel
Red channel
γ2-γ5
25 μm
G
Without DPM-Gal4
Light power (μW/mm²)
180
520
900
Odor
γ2-γ5
25 μm
H
With DPM-Gal4
180
γ2-γ5
25 μm
ACh3.0
ΔF/F0 -0.2
0.8
I
Light power (μW/mm²)
180
520
900
180
γ2-γ5 ΔF/F0
0.6
0.4
0.2
0.0
***
***
***
n.s.
n.s.
n.s.
Basal
Light
Basal
Light
Basal
Light
Odor
Light
DPM-Gal4
-
-
-
-
+
60 s light
60 s light
60 s light
10 s odor
60 s light
γ2-γ5
ACh3.0
ΔF/F0
10%
60 s
γ2-γ5
ACh3.0
ΔF/F0
10%
60 s
J
DPM > CsCh;
KC > ACh3.0; Trhn-/-
635 nm light
KC
DPM
γ lobe
635 nm light
CsCh
KC
DPM
ACh
K
Odor
Shock
Light off
Light on
Light off
Light on
γ1
γ2
γ3
γ4
γ5
γ lobe
25 μm
ACh3.0
ΔF/F0 0
1
ACh3.0
ΔF/F0 0
0.5
Phasic
20 s light
ACh3.0
γ lobe
ΔF/F0
20%
10 s
Phasic
5 s Odor
5 s Odor
0.5 s Shock
0.5 s Shock
L
Trhn-/-
Light off
Light on
γ1 phasic ΔF/F0
1.5
1.0
0.5
0.0
n.s.
n.s.
γ2-γ5 phasic ΔF/F0
0.8
0.6
0.4
0.2
0.0
**
n.s.
Odor
Shock

Figure S5. Optogenetically activating the DPM neuron inhibits both phasic and tonic release of ACh from KCs, related to Figure 4.

(**A**) Schematics depicting the *in vivo* imaging setup in which the CsChrimson-expressing DPM neuron was activated by light pulses (1 ms/pulse, 635 nm, 10 Hz), and ACh was measured in the γ lobe using ACh3.0 expressed in KCs.

(**B-C**) Representative pseudocolor images of the change in ACh3.0 fluorescence in response to odor (5-s application) or electric shock (0.5 s, 90 V) in the absence or presence of light stimulation (**B**), or to 60-s light stimulation in the absence or presence of 5-HT receptors' antagonists (**C**).

(**D-E**) **Left**: schematics depicting strategies for calculating the phasic ACh3.0 signals with different baselines. **Right**: summary of the phasic ACh3.0 signals measured in response to odor (1 s) or electric shock (0.5 s, 90 V) applied in the absence of presence of light stimulation; n=8-9 flies/group. Note that the summary data in (**D**) are reproduced from Figure 4I.

(**F-I**) Light stimulation does not affect ACh dynamics in flies with UAS-CsChrimson but without DPM-Gal4 driver, ruling out the unspecific effect caused by leaky expression of channelrhodopsin. Shown are schematics (**F, left**) and fluorescence images (**F, right**) depicting the *in vivo* imaging setup in which ACh was measured with ACh3.0 expressed in KCs, while the light pulses (5 ms/pulse, 635 nm, 10 Hz) were delivered to the brain of the fly only carrying UAS-CsCh-mCherry, but not DPM-Gal4. Also shown are representative pseudocolor images (**G, H, top**), average and individual traces (**G, H, bottom**), and group analysis (**I**) of the change in ACh3.0 fluorescence in response to light (60 s) with the indicated power or odor stimuli (10 s), in flies without or with DPM-Gal4. Note that the data of flies with DPM-Gal4 are reproduced from the saline group of Figures 4J-4K and S5C. Also note that light pulses with the power of 180 μW/mm^2 were used in Figures 4J-4K and S5C. The gap junction blocker CBX (100 μM) was present throughout the experiment.

(**J-L**) Optogenetically activating the DPM neuron-elicited decrease of the phasic ACh signal is diminished in Trhn$^{-/-}$ flies. Shown are schematics (**J**) depicting the *in vivo* imaging setup in which the CsChrimson-expressing DPM neuron was activated by light pulses (5 ms/pulse, 635 nm, 10 Hz), and ACh was measured in the γ lobe using ACh3.0 expressed in KCs of Trhn$^{-/-}$ flies. Also shown are representative pseudocolor images (**K, top**), average and individual traces (**K, bottom**), and group analysis (**L**) of the change in ACh3.0 fluorescence in response to odor (1 s) or electric shock (0.5 s, 90 V) in the absence or presence of light; n=6-12 flies/group. A fly received 2-8 pairs of odor and/or shock stimuli, and within each pair the light-on and light-off trials were performed in random order. Note that the gap junction blocker CBX (100 μM) was present throughout these experiments.

*p<0.05; **p<0.01; ***p<0.001; and n.s., not significant (paired or unpaired Student's t-test).

Figure S6

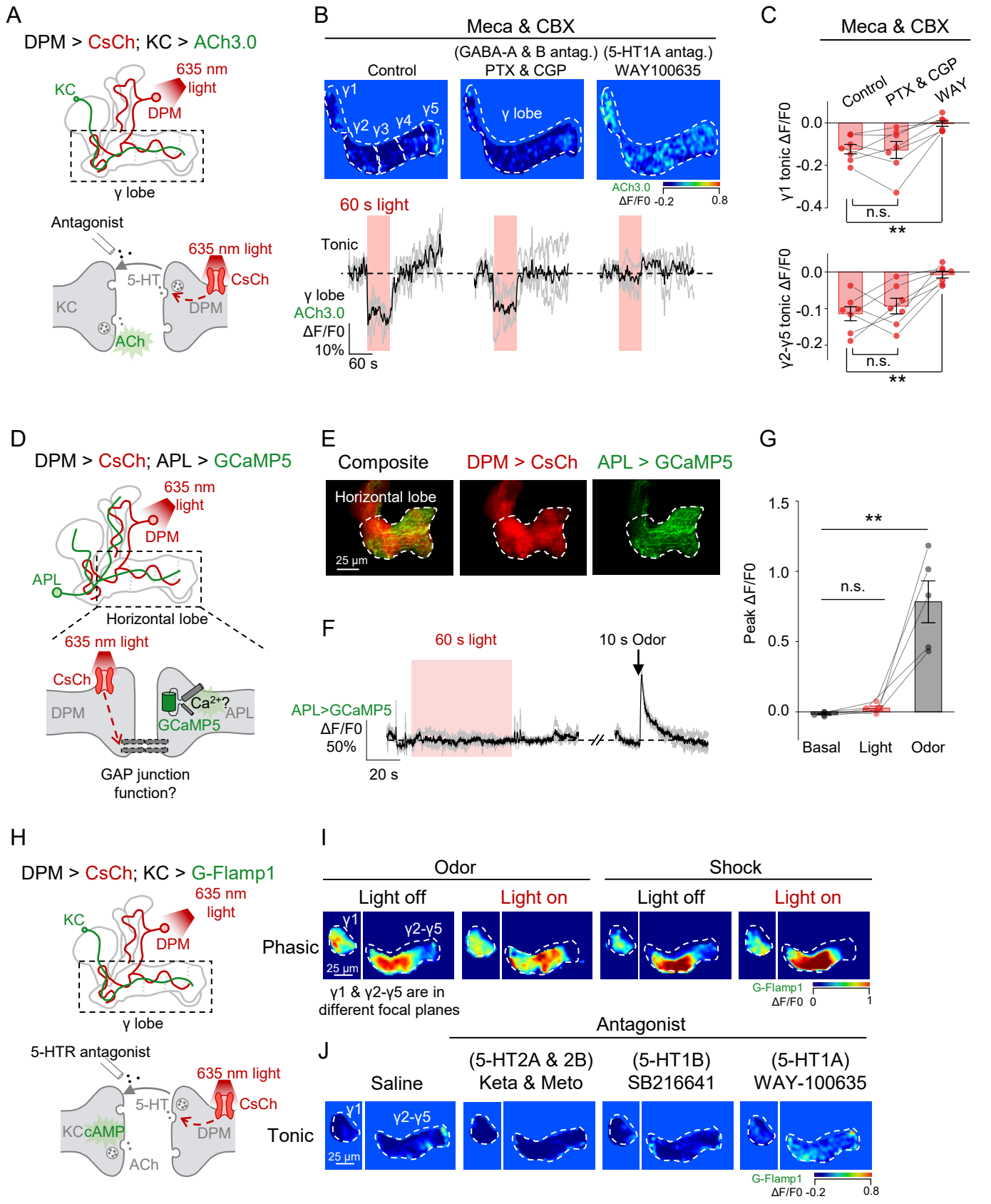

A
DPM > CsCh; KC > ACh3.0
635 nm light
KC
DPM
γ lobe
Antagonist
635 nm light
5-HT
CsCh
KC
DPM
ACh
B
Meca & CBX
(GABA-A & B antag.) (5-HT1A antag.)
Control
PTX & CGP
WAY100635
γ1
γ2
γ3
γ4
γ5
γ lobe
ACh3.0
ΔF/F0 -0.2
0.8
60 s light
Tonic
γ lobe
ACh3.0
ΔF/F0
10%
60 s
C
Meca & CBX
Control
PTX & CGP
WAY
γ1 tonic ΔF/F0
0.0
-0.2
-0.4
n.s.
**
γ2-γ5 tonic ΔF/F0
0.0
-0.1
-0.2
n.s.
**
D
DPM > CsCh; APL > GCaMP5
635 nm light
DPM
APL
Horizontal lobe
635 nm light
CsCh
DPM
Ca2+?
GCaMP5
APL
GAP junction function?
E
Composite
DPM > CsCh
APL > GCaMP5
Horizontal lobe
25 μm
F
60 s light
10 s Odor
APL>GCaMP5
ΔF/F0
50%
20 s
G
Peak ΔF/F0
1.5
1.0
0.5
0.0
**
n.s.
Basal
Light
Odor
H
DPM > CsCh; KC > G-Flamp1
635 nm light
KC
DPM
γ lobe
5-HTR antagonist
635 nm light
5-HT
CsCh
KCcAMP
DPM
ACh
I
Odor
Shock
Light off
Light on
Light off
Light on
Phasic
γ1
γ2-γ5
25 μm
γ1 & γ2-γ5 are in different focal planes
G-Flamp1
ΔF/F0 0
1
Antagonist
J
Saline
(5-HT2A & 2B) Keta & Meto
(5-HT1B) SB216641
(5-HT1A) WAY-100635
Tonic
γ1
γ2-γ5
25 μm
G-Flamp1
ΔF/F0 -0.2
0.8

Figure S6. Optogenetically activating the DPM neuron inhibits ACh and cAMP signals of the KCs, but does not affect the Ca^{2+} signal of the APL neuron, related to Figure 4.

(**A-C**) DPM activation-induced inhibitory effect on the tonic ACh signal is mediated by 5-HT, instead of GABA. Shown are schematics (**A**) depicting the *in vivo* imaging setup in which the CsChrimson-expressing DPM neuron was activated by light pulses (5 ms/pulse, 635 nm, 4 Hz), and ACh was measured in the γ lobe using ACh3.0 expressed in KCs in the absence or presence of antagonists of GABA and 5-HT receptors. Also shown are representative pseudocolor images (**B, top**), average and individual traces (**B, bottom**), and summary (**C**) of the change in ACh3.0 fluorescence in response to a 60-s light pulse. Where indicated, the GABA-A receptor antagonist picrotoxin (PTX, 125 μM), the GABA-B receptor antagonist CGP54626 (CGP, 25 μM), and the 5-HT1A receptor antagonist WAY-100635 (WAY, 20 μM) were applied; n=7 flies/group. For each fly, the experiment was divided into 3 sessions, and each session contains 3 repetitive trials. Note that the nAChR antagonist Meca (30 μM) and the gap junction blocker CBX (100 μM) were present throughout the imaging experiments.

(**D-G**) Optogenetically activating the DPM neuron does not cause significant effect on the Ca^{2+} signal of the APL neuron. Shown are schematics (**D**) and representative fluorescence images (**E**) of the *in vivo* imaging setup, in which the CsChrimson-expressing DPM neuron was activated by light (1 ms/pulse, 635 nm, 10 Hz), and the Ca^{2+} signal was measured using GCaMP5 expressed in the APL neuron. Also shown are average and individual traces (**F**), and summary (**G**) of the change in GCaMP5 fluorescence in response to light (60 s) or odor (10 s); n= 5 flies/group. Each fly received 3 trials of light stimulation and 3 trials of odor stimulation in random order.

(**H-J**) Activating the DPM neuron only inhibits tonic cAMP dynamics, but does not influence phasic cAMP increase evoked by odor or shock stimuli in the γ lobe. Shown are schematics (**H**) depicting the *in vivo* imaging setup in which the CsChrimson-expressing DPM neuron was activated by light pulses (5 ms/pulse, 635 nm, 4 Hz) and cAMP was measured in the γ lobe using G-Flamp1 expressed in KCs. Also shown are representative pseudocolor images of the change in G-Flamp1 fluorescence in response to odor (5-s application) or electric shock (0.5 s, 90 V) in the absence or presence of light stimulation (**I**), or to 60-s light stimulation in the absence or presence of 5-HT receptors' antagonists applied at 20 μM (**J**).

$^{**}p<0.01$ and n.s., not significant (paired Student's *t*-test).

Figure S7

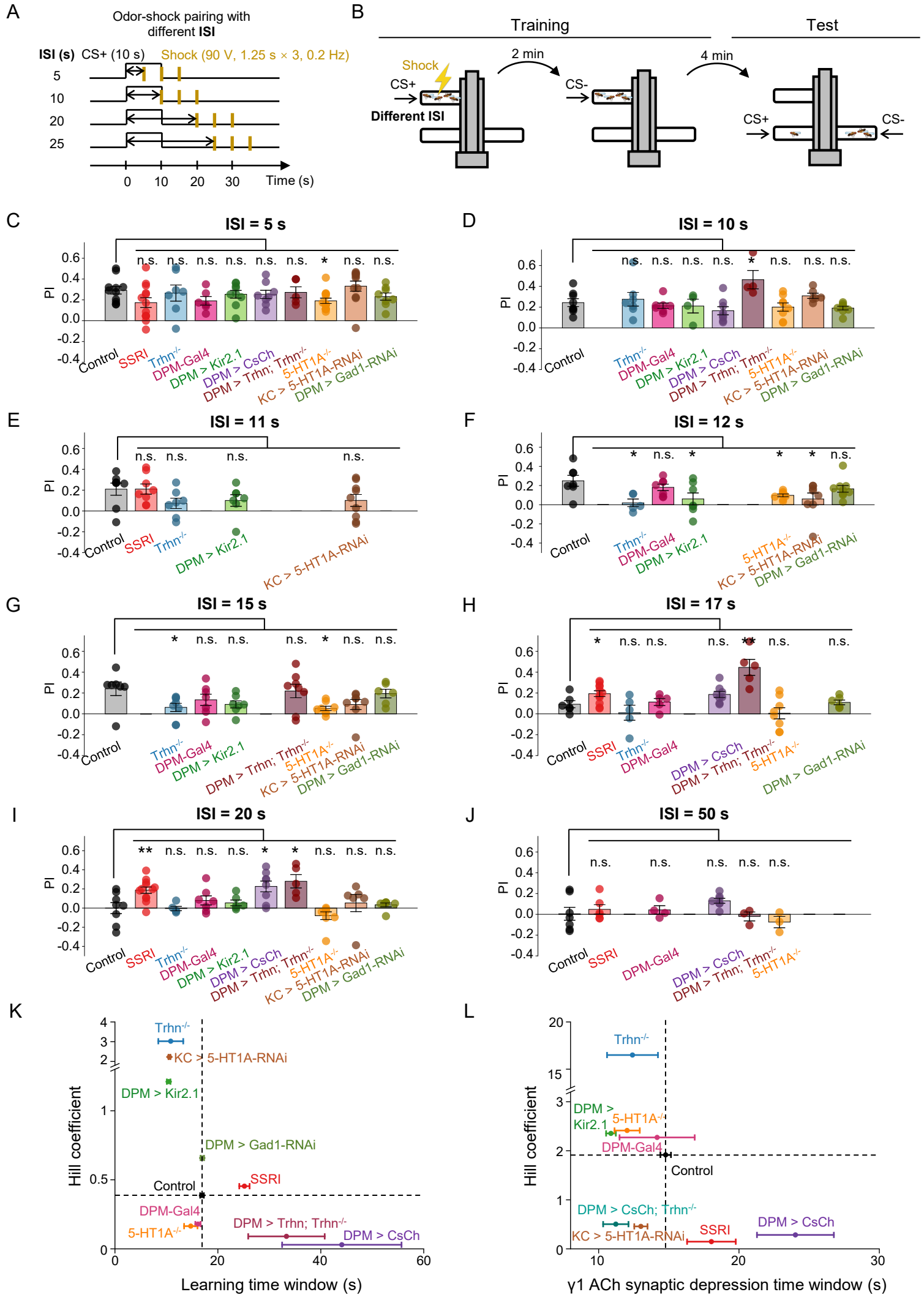

A
Odor-shock pairing with different ISI
ISI (s) CS+ (10 s) Shock (90 V, 1.25 s × 3, 0.2 Hz)
5
10
20
25
0 10 20 30 Time (s)
B
Training
Test
Shock
CS+
Different ISI
2 min
CS-
4 min
CS+
CS-
C
ISI = 5 s
D
ISI = 10 s
E
ISI = 11 s
F
ISI = 12 s
G
ISI = 15 s
H
ISI = 17 s
I
ISI = 20 s
J
ISI = 50 s
PI
Control
SSRI
Trhn-/-
DPM-Gal4
DPM > Kir2.1
DPM > CsCh
DPM > Trhn; Trhn-/-
5-HT1A-/-
KC > 5-HT1A-RNAi
DPM > Gad1-RNAi
K
Hill coefficient
Learning time window (s)
L
Hill coefficient
γ1 ACh synaptic depression time window (s)

Figure S7. Summary of the PIs, Hill coefficients and coincidence time windows, related to Figure 8.

(**A**-**B**) Schematics depicting the protocol for odor-shock pairing with varying ISIs (**A**) and the T-maze assay for measuring the olfactory memory (**B**).

(**C-J**) Summary of the PIs measured using the indicated ISI of flies with various genetic or pharmacological manipulations; n=3-11 for each group.

(**K-L**) Correlation analysis between the Hill coefficient and the length of coincidence time window (t_{50} ± standard error) of flies with various genetic or pharmacological manipulations. The dashed lines indicate the values of control flies.

Note that the data in (**C-K**) are reproduced from Figures 1 and 7, and data in (**L**) are reproduced from Figures 2 and 5.

*p<0.05; **p<0.01; and n.s., not significant (unpaired Student's t-test).

Figure S8

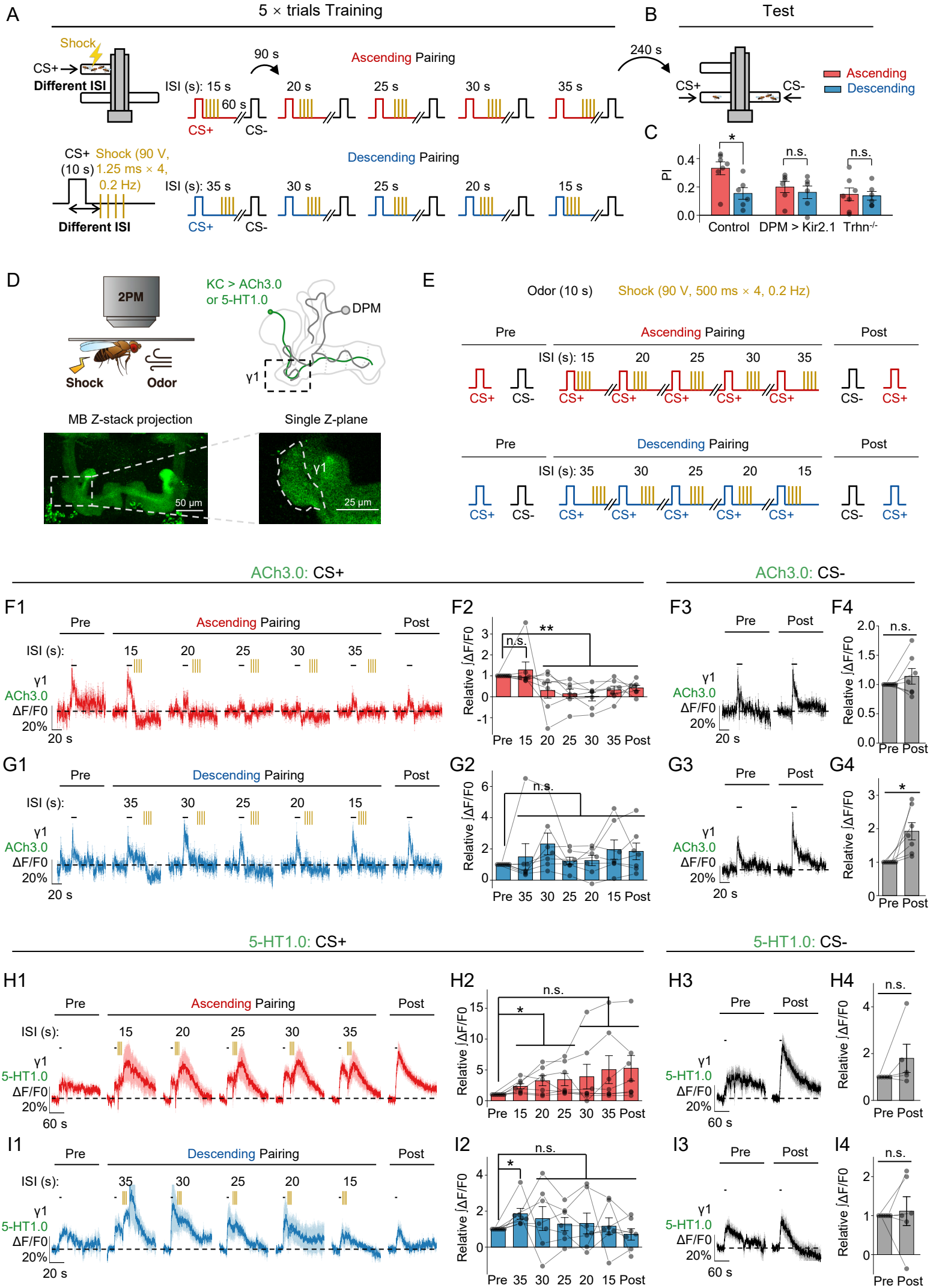

A
5 × trials Training
Shock
CS+
Different ISI
90 s
Ascending Pairing
ISI (s): 15 s
20 s
25 s
30 s
35 s
60 s
CS+
CS-
240 s
CS+ Shock (90 V, (10 s) 1.25 ms × 4, 0.2 Hz)
Descending Pairing
ISI (s): 35 s
30 s
25 s
20 s
15 s
Different ISI
B
Test
CS+
CS-
Ascending
Descending
C
PI
0.4
0.2
0.0
*
n.s.
n.s.
Control
DPM > Kir2.1
Trhn-/-
D
2PM
Shock
Odor
KC > ACh3.0 or 5-HT1.0
DPM
γ1
MB Z-stack projection
Single Z-plane
50 μm
25 μm
E
Odor (10 s)
Shock (90 V, 500 ms × 4, 0.2 Hz)
Pre
Ascending Pairing
Post
ISI (s): 15
20
25
30
35
CS+
CS-
Pre
Descending Pairing
Post
ISI (s): 35
30
25
20
15
CS+
CS-
ACh3.0: CS+
ACh3.0: CS-
F1
Pre
Ascending Pairing
Post
ISI (s):
15
20
25
30
35
γ1
ACh3.0
ΔF/F0
20%
20 s
F2
Relative ∫ΔF/F0
**
n.s.
Pre 15 20 25 30 35 Post
F3
Pre
Post
γ1
ACh3.0
ΔF/F0
20%
20 s
F4
Relative ∫ΔF/F0
n.s.
Pre Post
G1
Pre
Descending Pairing
Post
ISI (s):
35
30
25
20
15
γ1
ACh3.0
ΔF/F0
20%
20 s
G2
Relative ∫ΔF/F0
n.s.
Pre 35 30 25 20 15 Post
G3
Pre
Post
γ1
ACh3.0
ΔF/F0
20%
20 s
G4
Relative ∫ΔF/F0
*
Pre Post
5-HT1.0: CS+
5-HT1.0: CS-
H1
Pre
Ascending Pairing
Post
ISI (s):
15
20
25
30
35
γ1
5-HT1.0
ΔF/F0
20%
60 s
H2
Relative ∫ΔF/F0
n.s.
*
Pre 15 20 25 30 35 Post
H3
Pre
Post
γ1
5-HT1.0
ΔF/F0
20%
60 s
H4
Relative ∫ΔF/F0
n.s.
Pre Post
I1
Pre
Descending Pairing
Post
ISI (s):
35
30
25
20
15
γ1
5-HT1.0
ΔF/F0
20%
20 s
I2
Relative ∫ΔF/F0
n.s.
*
Pre 35 30 25 20 15 Post
I3
Pre
Post
γ1
5-HT1.0
ΔF/F0
20%
60 s
I4
Relative ∫ΔF/F0
n.s.
Pre Post

Figure S8. 5-HT from the DPM neuron helps to bridge the CS-US temporal gap, related to Figure 8.

(**A-C**) Schematics depicting different protocols for ascending and descending pairing paradigms (**A-B**) and summary of PIs (**C**) measured in control flies, DPM > Kir2.1 flies, and $Trhn^{-/-}$ flies; n=6-7 for each group.

(**D-E**) Schematics depicting the *in vivo* two-photon imaging setup (**D, top**), fluorescence images (**D, bottom**), and the ascending and descending pairing protocols (**E**) for measuring the change in ACh or 5-HT dynamics in the MB γ1 compartment with ACh3.0 or 5-HT1.0 expressed in KCs.

(**F-G**) Shown are average (±SEM) traces and summary of ACh release measured in the ascending (**F**) or descending (**G**) pairing paradigms in response to CS+ or CS-; n=6-7 flies/group.

(**H-I**) Similar to (**F-G**), except that 5-HT dynamics were measured and analyzed.

**p*<0.05; ***p*<0.01; and n.s., not significant (paired or unpaired Student's *t*-test).

SUPPLEMENTAL REFERENCES

1. Scheffer, L.K., Xu, C.S., Januszewski, M., Lu, Z., Takemura, S.Y., Hayworth, K.J., Huang, G.B., Shinomiya, K., Maitlin-Shepard, J., Berg, S., et al. (2020). A connectome and analysis of the adult Drosophila central brain. Elife 9. 10.7554/eLife.57443.
2. Aso, Y., Ray, R.P., Long, X., Bushey, D., Cichewicz, K., Ngo, T.T., Sharp, B., Christoforou, C., Hu, A., Lemire, A.L., et al. (2019). Nitric oxide acts as a cotransmitter in a subset of dopaminergic neurons to diversify memory dynamics. Elife 8. 10.7554/eLife.49257.