Figure S1: Further anatomical analysis of 71D08-LexA and 37G11-GAL4

(A) Partial Z-projection of R71D08-LexA driving GFP. Expression of MBON-α2sc is observed in the dorsal LH and along the vertical lobe of the MB. Representative image of n=3 stacks. (B-C) Immunohistochemistry of PD2a1/b1 to determine neurotransmitter. (B) Colocalization of GABA (magenta) and R37G11-GAL driving GFP (green) expression indicates that PD2a1/b1 neurons are not GABAergic. (C) Colocalization of GABA (magenta) and R37G11-GAL driving GFP (green) expression indicates that PD2a1/b1 neurons are not GABAergic. (D) Colocalization of VGlut (magenta) and split-GAL4 line LH989 driving mVenus (green) expression indicates that PD2a1/b1 neurons are not glutamatergic (yellow arrows label sample bouton). For B-C the left panel is a composite while the middle and right panels represent single channels. The scale bar for A is 30µm and 5µm for B-C. For B-C, each image is a representative slice from n=4 brains.

Figure S2: Anatomical and behavioural analysis of two other cell-types identified in the overlap screen

(A) A segmented image of AV6a1. (B) A segmented image of the identified SIP cell-type. (C) Z-projection of double labelling between AV6a1 dendrites and MBON-α2sc. MBON-α2sc presynapses are labelled with Brp(d3)::mCherry (magenta) while AV6a1 is labelled with membrane-bound GFP (in green). (D) Z-projection of double labelling between AV6a1 dendrites and MBON-α2sc. The MBON-α2sc presynapses are labelled with Brp(d3)::mCherry (magenta) while the SIP neuron is labelled with membrane-bound GFP (in green). Note that this LexA line contains both MBON-α2sc (dorsal) and MBON-α′3ap (ventral) axons (E) Unsegmented Z-projection of identified AV6a1 GAL4 line, Insite0089-GAL4, driving CD8::GFP (Green) with an nc82 counterstain (magenta). Representative stack from n=3 brains. This brain is registered to the JFRC2 template brain. (F) Unsegmented Z-projection of identified SIP GAL4 line, R58G03-GAL4, driving CD8::GFP (Green) with an nc82 counterstain (magenta). This brain is registered to the JFRC2 template brain. Data from Virtual Fly Brain (http://www.virtualflybrain.org/site/vfb_site/home.htm) (G) Flies expressing Shi^{ts} driven by Insite0089-GAL4, and their genotypic controls, were trained with a single-cycle protocol at permissive temperature and tested 3h later at restrictive temperature. There was no significant difference in memory performance between the three groups ($n = 17$, $F_{(2,50)} = 2.29$, $p = 0.11$). (H) Flies expressing Shi^{ts} driven by R58G03-GAL4, and their genotypic controls, were trained with a single-cycle protocol at permissive temperature and tested 3h later at restrictive temperature.

There was no significant difference in memory performance between the three groups (n = 14, $F_{(2,41)} = 1.61$, p = 0.21). Data are presented as mean±SEM. Scale bar is 30μm for A-B and E-F, 5μm for C-D.

Figure S3: Permissive temperature controls for 37G11-GAL4 memory retrieval experiments

When assayed at the permissive temperature, flies expressing Shi^{ts} through R37G11-GAL4 driver performed similarly to their controls for immediate memory (A: n = 8, $F_{(2,23)} = 3.52$, p = 0.048, no difference between groups in pairwise comparisons), 3h-memory (B: n = 16, $F_{(2,47)} = 4.23$, p = 0.02, no difference between +/UAS-Shi^{ts} and 37G11>UAS-Shi^{ts} groups in pairwise comparisons), and long-term memory (C: n = 9–10, $F_{(2,28)} = 0.53$, p = 0.59). Data are presented as mean±SEM.

Figure S4: Permissive temperature controls for split-GAL4 memory retrieval experiments

When assayed at the permissive temperature, flies expressing Shi^{ts} through LH989 and LH991 performed similarly to their controls for immediate memory after 1x training (A: LH991, n = 12, $F_{(2,35)} = 0.14$, p = 0.87; B: LH989, n = 12–13, $F_{(2,36)} = 3.66$, p = 0.037, no difference between 37G11/+ and 37G11>UAS-Shi^{ts} groups in pairwise comparisons), for 3h-memory after 1x training (C: LH991, n = 15, $F_{(2,44)} = 0.28$, p = 0.76; D: LH989, n = 10, $F_{(2,29)} = 1.79$, p = 0.19), and for long-term memory after spaced training (E: LH991, n = 17, $F_{(2,47)} = 0.27$, p = 0.76; F: LH989, n = 7–9, $F_{(2,24)} = 2.10$, p = 0.15). Data are presented as mean±SEM.

Figure S5: Single-cell analysis of PD2a1/b1 GAL4 and split-GAL4 lines

(A) An example a MultiColor FlipOut (MCFO) experiment. A single neuron is labelled in green while the counterstain is nc82 (magenta). The counterstain is registered to the JFRC2013 template brain (B) Skeletonized representations of all 22 single-neurons derived from MCFO registered to JFRC2013 (grey). (C-D) More detailed view of all 22 skeletons from an anterior (C) and dorsal (D) view. For C-D, only the LH (red) and calyx (blue) neuropil regions are labelled. Scale bar is 30μm.

Figure S6: Effectors in landing site VK5 are driven in MBON-α2sc and MBON-α′3ap by 71D08-LexA

Confocal z-projection of R71D08-LexA driving expression of TdTomato (green) in landing site VK5. Expression recapitulates that of the R71D08 enhancer including MBON-α2sc and MBON-α′3ap. Counterstain is nc82 (magenta). Representative stack of n=3 brains.

Figure S7: Electron microscopy reconstruction strategy and confirmation that identified cells are PD2a1/b1

(A) Cross-section of the PD2a1/b1 tract in the electron microscopy dataset. Neurons confirmed to be PD2a1/b1 are labelled in light (PD2a1) and dark green (PD2b1). Possible primary neurites in the tract are labelled with a purple dot (178). The x,y,z position of the tract in the ssTEM whole brain volume is 482684, 206960, 151200 nm, section 4320. (B'-B'') Dorsal view of PD2a1 and PD2b1 skeletons from MCFO data (blue) and EM reconstructions (green), shown with the LH (green), CA (purple), SIP (yellow) and SMP (orange). Black line bisecting PD2 primary neurite tract indicate position of EM section in (A). Slight offset between light and EM is thought to be a product of registration error (C-C') NBLAST clustering PD2a1 and PD2b1 arbors from EM reconstructed, FlyCircuit and MCFO data using only their axonal compartments (C) or dendritic compartments (C') Skeletons are colored by the group to which they belong in the dendrogram. In (C) a1 and b1 neurons cluster apart. EM neurons are more similar to each other likely because they are from the same brain whereas the MCFO and FlyCircuit data are from different brains, and have been co-registered. In (C) the main two subdivisions denote the two routes PD2a1/b1 axons take around the MB (purple) vertical lobe. Labels name include the split-Gal4 line that generated the MCFO, or the Gal4 line driven by a neurotransmitter synthesis/processing protein used to generate the FlyCircuit flip-out data, or indicate an EM neuron in Figure 4B''.

Figure S8: Summary Electron Microscopy data for MBON-α2sc and PD2a1/b1

(A) Bar chart showing the neuropils targeted by the ipsilateral 'fly's right' MBON-α2sc axon, showing the postsynapses, presynapses and cable that the MBON has in each neuropil. Inset, presynapses across the MBON axon shown coloured by neuropil location. (B) The neuropils targeted by whole 'fly's right' PD2a1/b1 neurons and the PD2a1/b1 postsynapses, presynapses and cable length in each. Inset, postsynapses and presynapses across the PD2a1/b1 neurons

shown coloured by neuropil location. (C) Stripchart shows the cable length in µms for PD2a1/b1 dendrites, PD2b1 dendrites and PDa1/b1 axons. Stacked bar chart shows the proportion of cable and postsynapses that were found on microtubule-containing backbone and microtubule free 'twigs'. Inset, visualisation of backbone (red) and twigs (green) in PD2a1#1. (D) Number of presynapses and postsynapses plotted for the presumptive axon and dendrite of individual PD2a1 and PD2b1 neurons. The different categories and region of the cell are represented by distinct colors (PD2a1 or PD2b1) and shapes (Calyx, LH or whole compartments). Grey crosses indicate the mean. LH=lateral horn, MB_CA_R = mushroom body calyx, SIP=superior intermediate protocerebrum, SLP=superior lateral protocerebrum, SMP=superior medial protocerebrum, CRE = crepine, SCL = superior clamp, _R = fly's right (ipsilateral), _L = fly's left (contralateral).

Figure S9: Schematics for live calcium imaging training paradigms

Schematics of the 3 hour conditioning protocols used for *in vivo* imaging of odor responses after training with Oct (red) as CS+ (A) or Mch (blue) as CS+ (B). Left represents the actual training paradigm where electric shock (orange spikes) is presented alongside the CS+. Right panel represents the control unpaired protocol where electric shock is presented two minutes before olfactory stimulation. (C) Each of the training paradigm in A-B is considered a single unit which is used to space train flies to generate LTM for imaging 24 hours after training (left panel). The training paradigm described in A or B (depending on CS+) is repeated 5 times with an intertrial interval (ITI) of 15 minutes. For the unpaired control at 24 hours (right panel), the unpaired protocols from A or B (depending on which odor is CS+) is repeated 5 times with an intertrial interval (ITI) of 15 minutes.

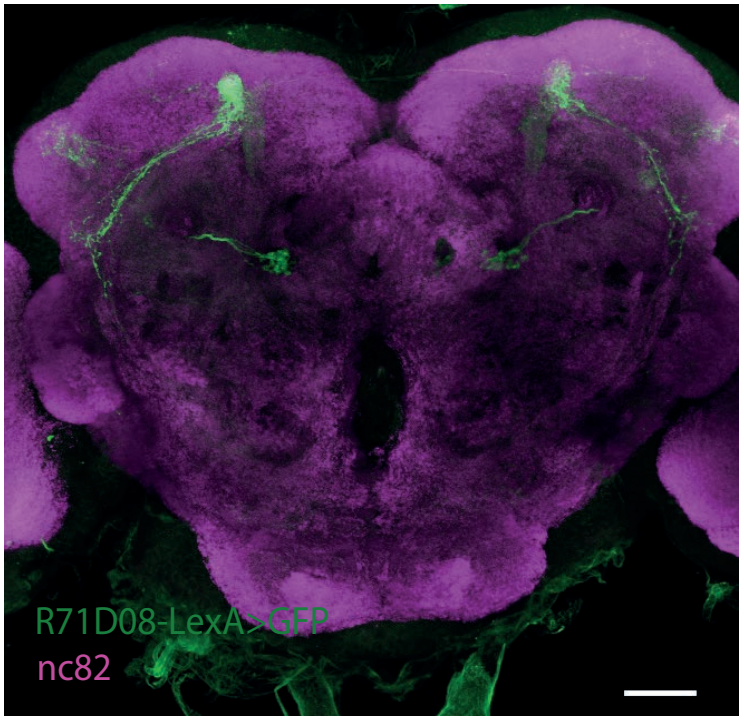
Figure S10: Structure of inputs onto PD2a1/b1 dendrites

(A-A') Distribution of synaptic sites in the three PD2a1/b1 neurons with the greatest absolute MBON-α2sc input. (A) Neurons shown with their branches colored by Strahler stream order, lower order branches in hotter colors. Stripcharts show the location of synapses on the PD2a1/b1 neurons' arbor, distance from primary axon-dendrite branch-point plotted against Strahler order (columns colored according to neuron visualisations above). Each column is split into synapses on microtubule-lacking twigs (open circles) and on microtubule containing backbone (closed circles). Point colors, light purple = left-side MBON-α2sc**,** purple = right-side MBON-α2sc, dark cyan = MBON-α'2, orange = PNs, grey = unknown, red = presynapses. (A') Neurons broken down into separate branches by amalgamating connected neurite of Strahler order 3 or smaller.

Separate branches shown in separate colors, the hotter the colors the larger the branch in terms of cable length. Branches with >20% MBON input highlighted in black. Stacked bar chart shows the percentage of postsynapses supplied by different inputting neurons. Bars ordered by branch length, branches of less than 10 μm pooled into one stacked bar denoted by grey dot. Numbers above bars indicate the number of synapses in each branch. (B) PNs strongly inputting PD2a1/b1 lack sister PNs and are sparsely responsive to odorants. Scatter plot of the number of connections by which selected groups of PNs input PD2a1/b1 neurons against the number of PNs in each glomerular group. Labels refer to cognate AL glomeruli. Number of PNs refers to the number of uniglomerular, excitatory PNs found in the EM whole brain volume. Size of circular points corresponds to the number of monomolecular odours, out of a battery of 31, to which PN dendrites are significantly responsive in a calcium imaging study (Badel et al. 2016). Triangles denote neurons not in the NP225-Gal5 line used in that study. Colors represent presumptive behavioural significance.

Figure S1

A



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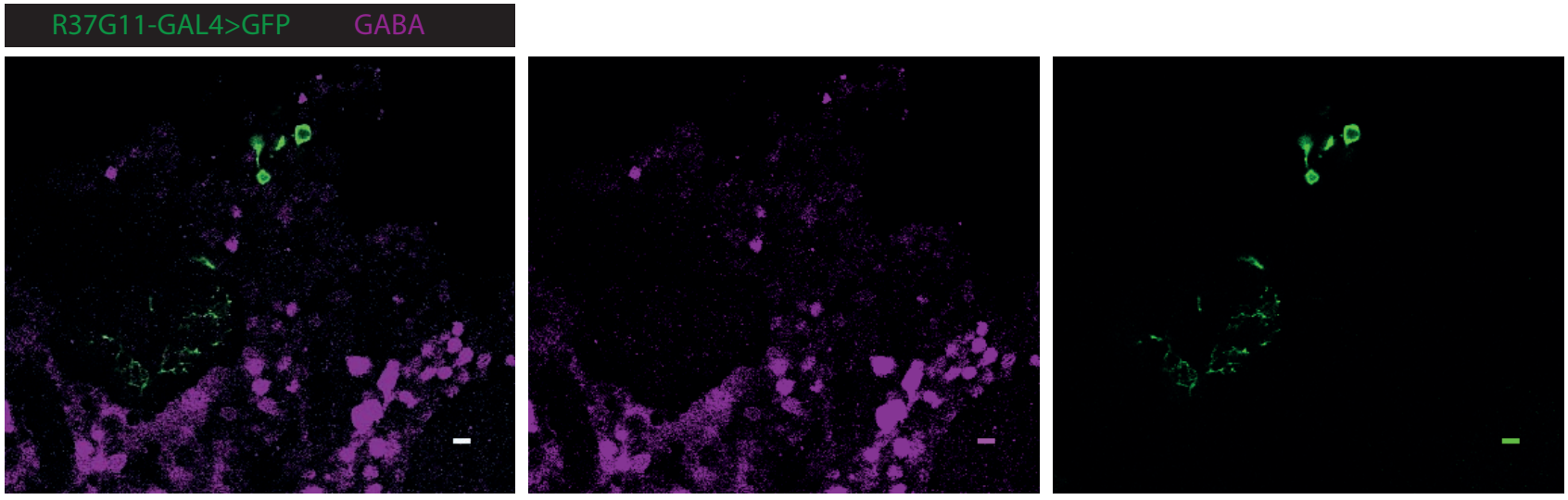

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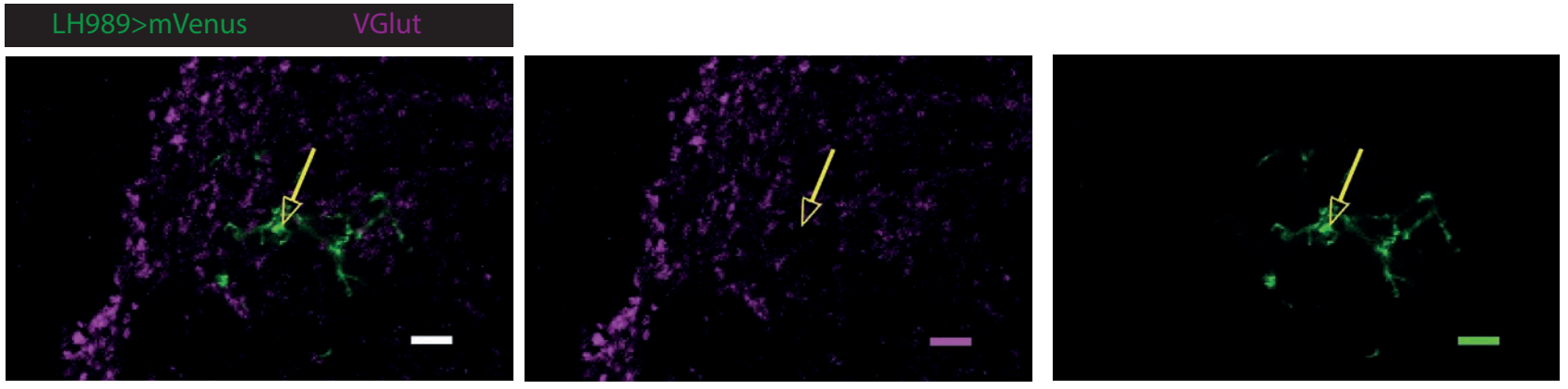


Figure S2

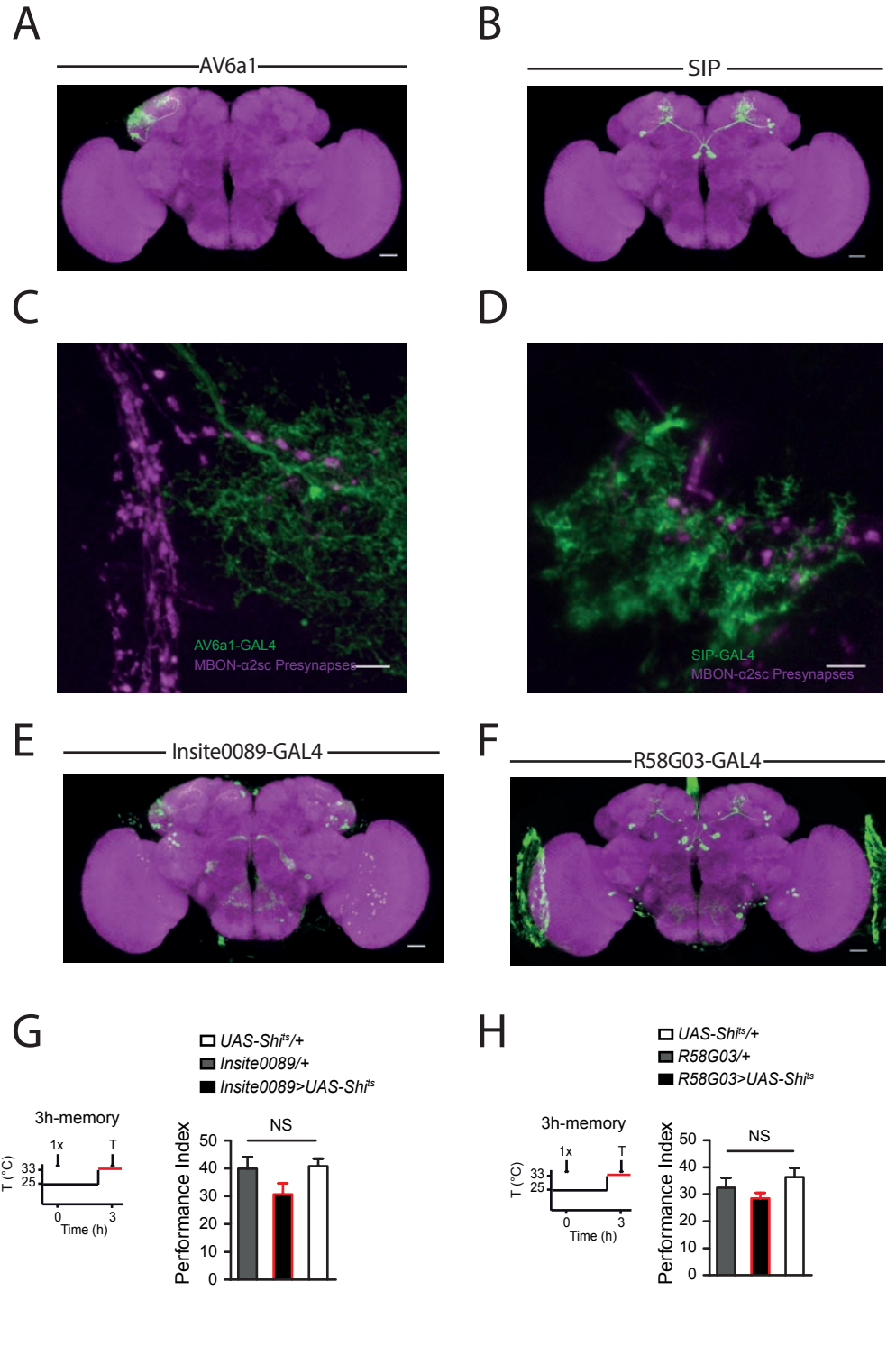

A
AV6a1
B
SIP
C
AV6a1-GAL4
MBON-α2sc Presynapses
D
SIP-GAL4
MBON-α2sc Presynapses
E
Insite0089-GAL4
F
R58G03-GAL4
G
UAS-Shits/+
Insite0089/+
Insite0089>UAS-Shits
3h-memory
1x
T
T (°C)
33
25
0
3
Time (h)
NS
Performance Index
50
40
30
20
10
0
H
UAS-Shits/+
R58G03/+
R58G03>UAS-Shits
3h-memory
1x
T
T (°C)
33
25
0
3
Time (h)
NS
Performance Index
50
40
30
20
10
0

Figure S3

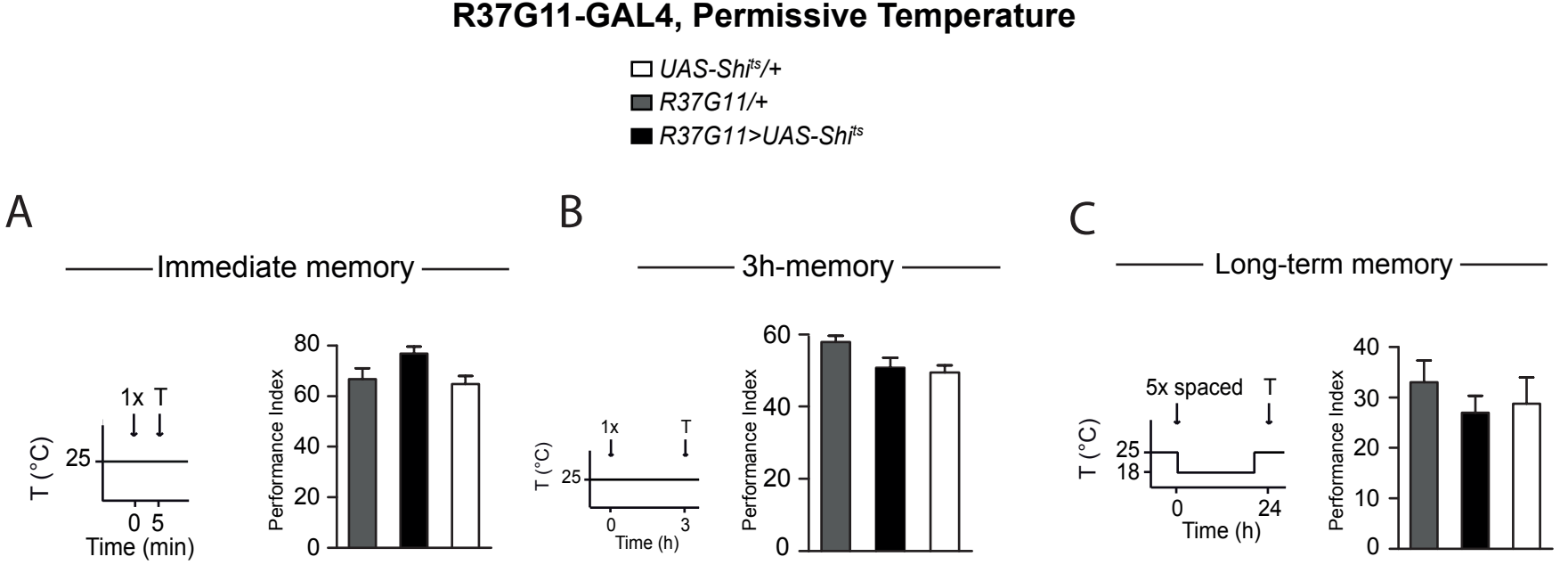

R37G11-GAL4, Permissive Temperature
UAS-Shi^ts/+
R37G11/+
R37G11>UAS-Shi^ts
A
Immediate memory
1x T
T (°C)
25
0 5
Time (min)
Performance Index
80
60
40
20
0
B
3h-memory
1x
T
T (°C)
25
0
3
Time (h)
Performance Index
60
40
20
0
C
Long-term memory
5x spaced
T
T (°C)
25
18
0
24
Time (h)
Performance Index
40
30
20
10
0

Figure S4

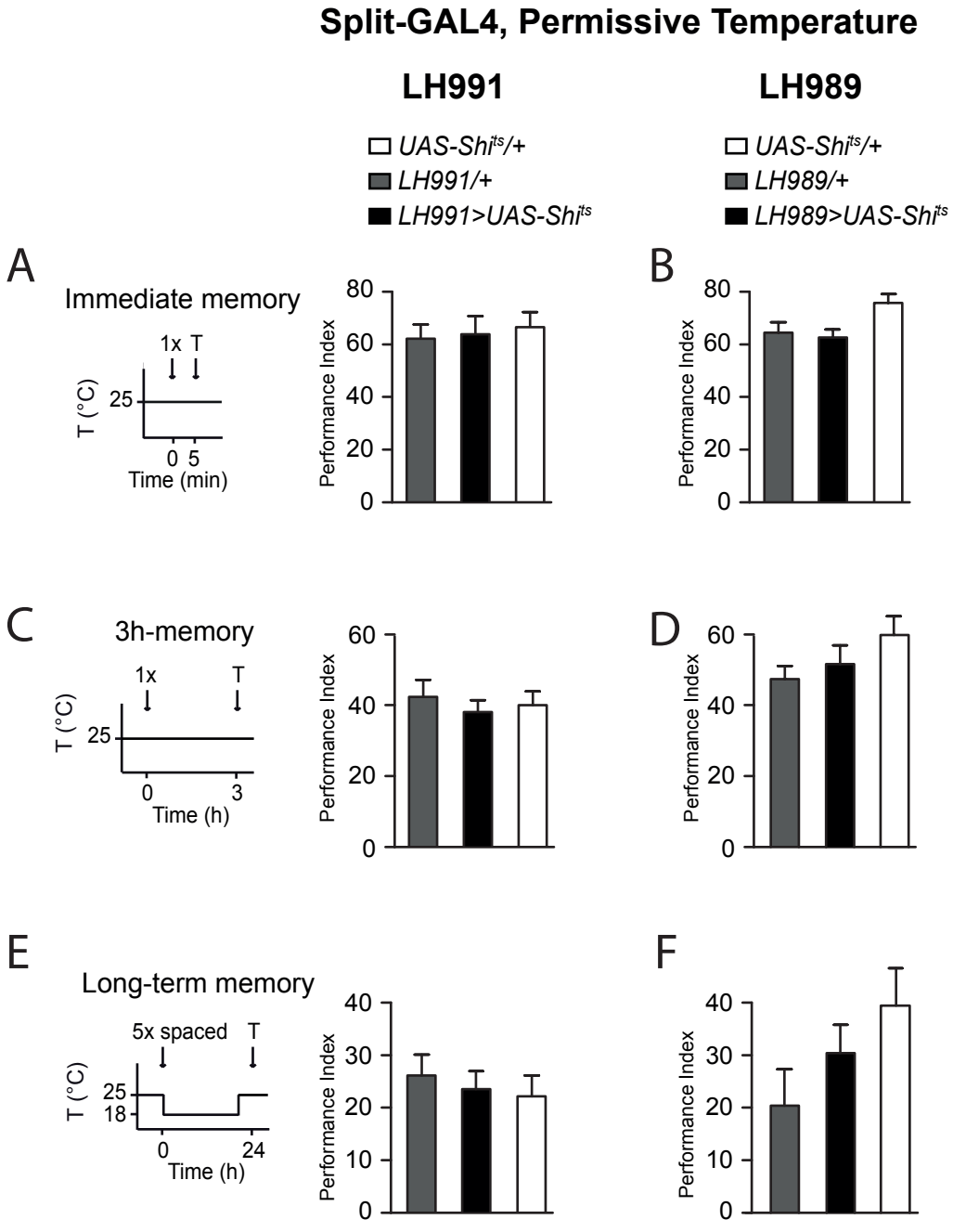
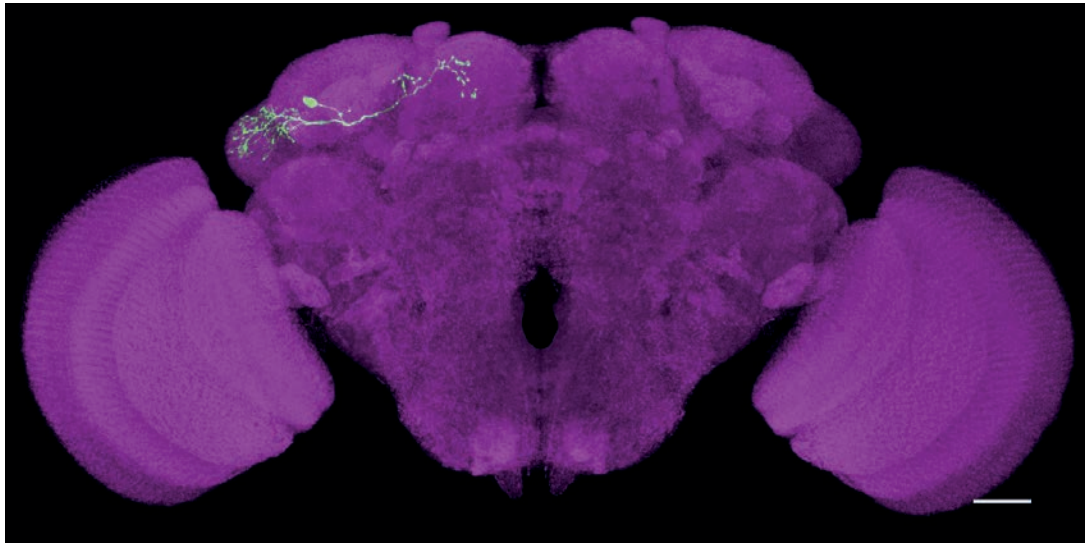

Split-GAL4, Permissive Temperature
LH991
LH989
UAS-Shi^ts/+
LH991/+
LH991>UAS-Shi^ts
UAS-Shi^ts/+
LH989/+
LH989>UAS-Shi^ts
A
Immediate memory
1x T
T (°C)
25
0 5
Time (min)
Performance Index
80
60
40
20
0
B
C
3h-memory
1x
T
0
3
Time (h)
D
E
Long-term memory
5x spaced
18
24
F

Figure S5

A



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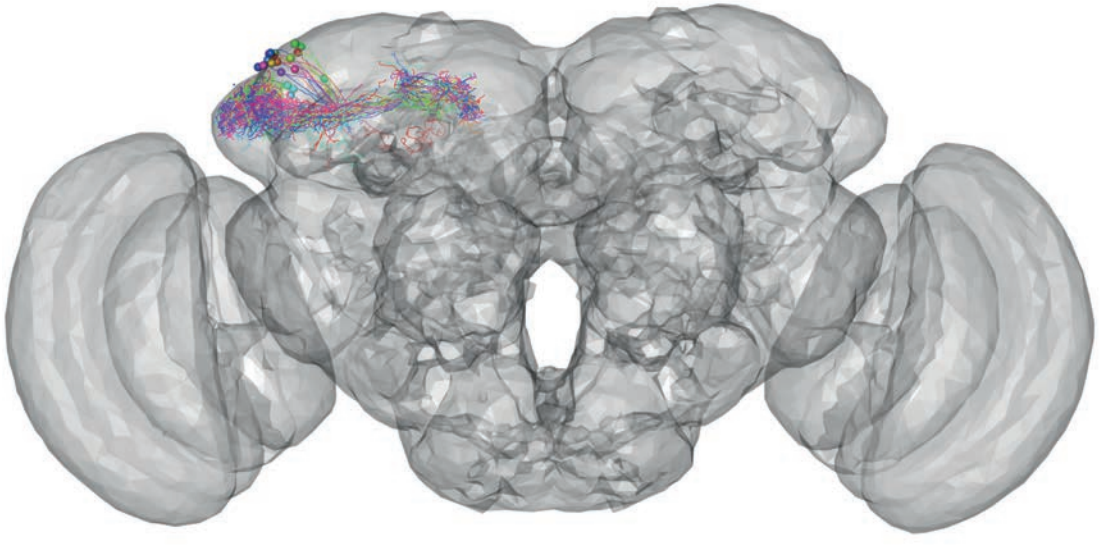

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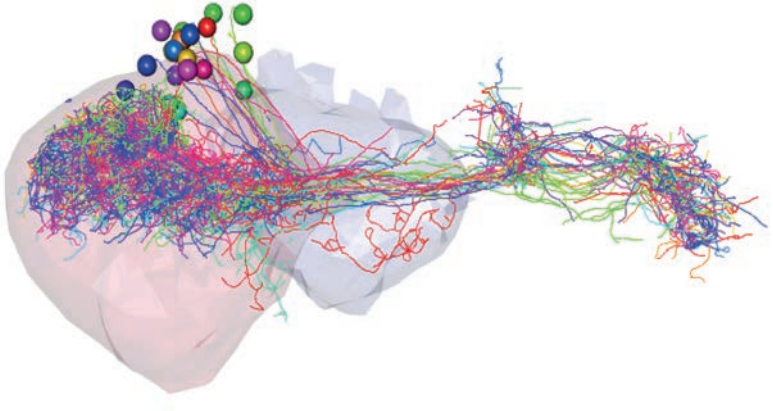

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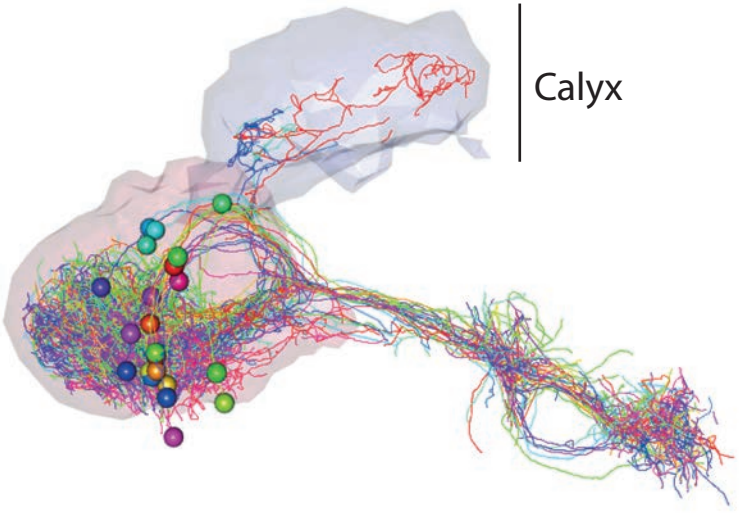


Figure S6

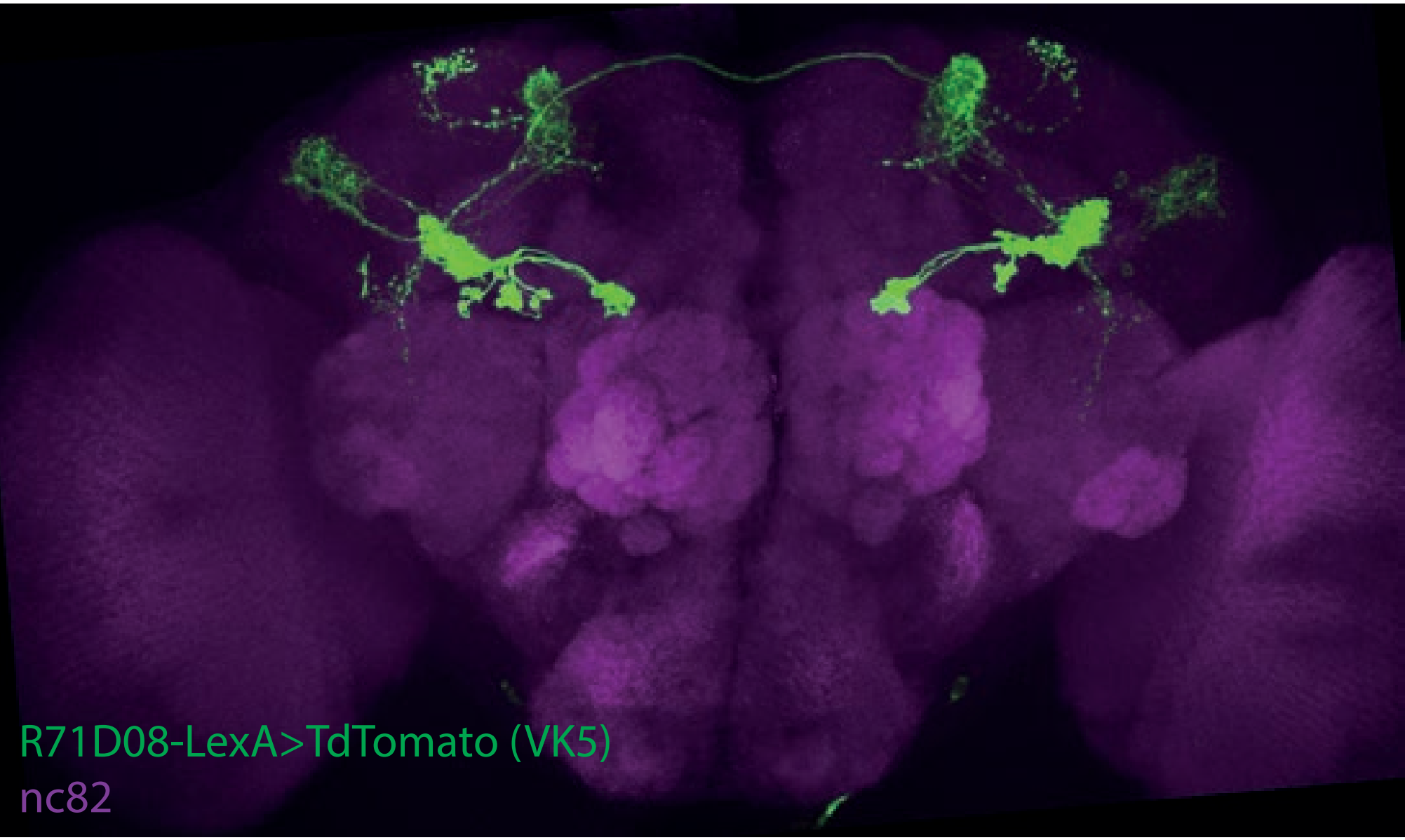
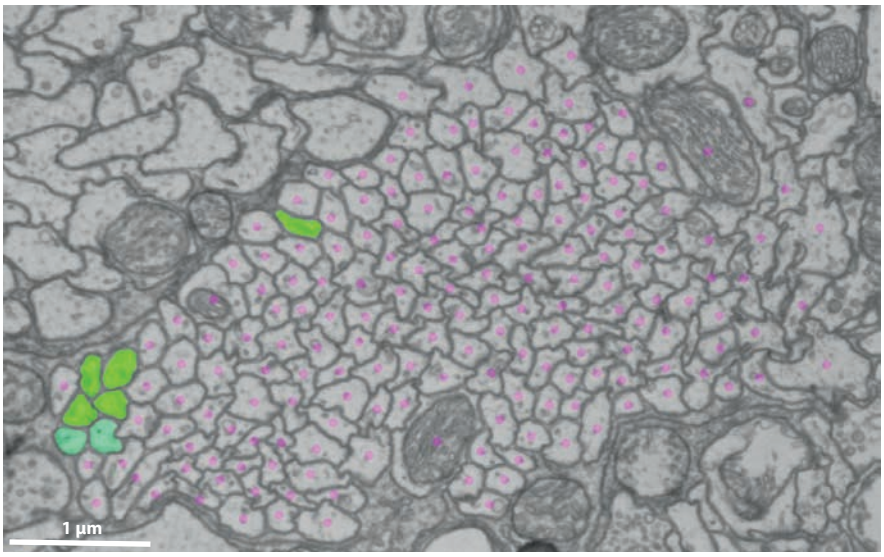


Figure S7

A



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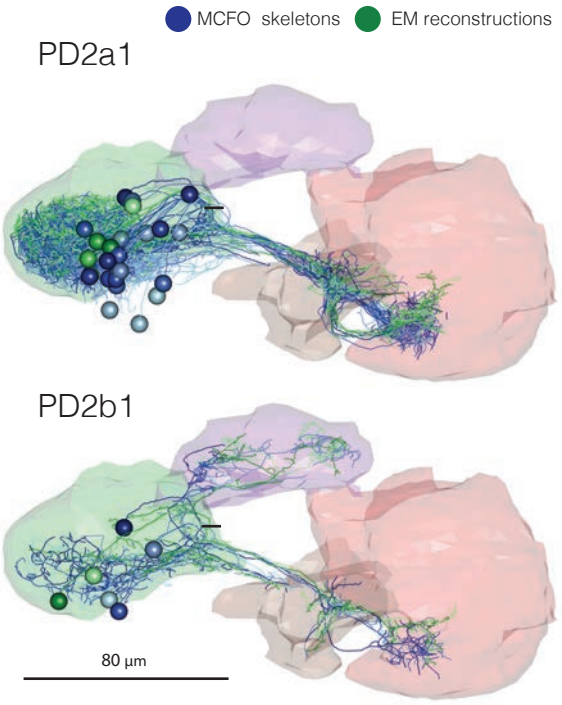

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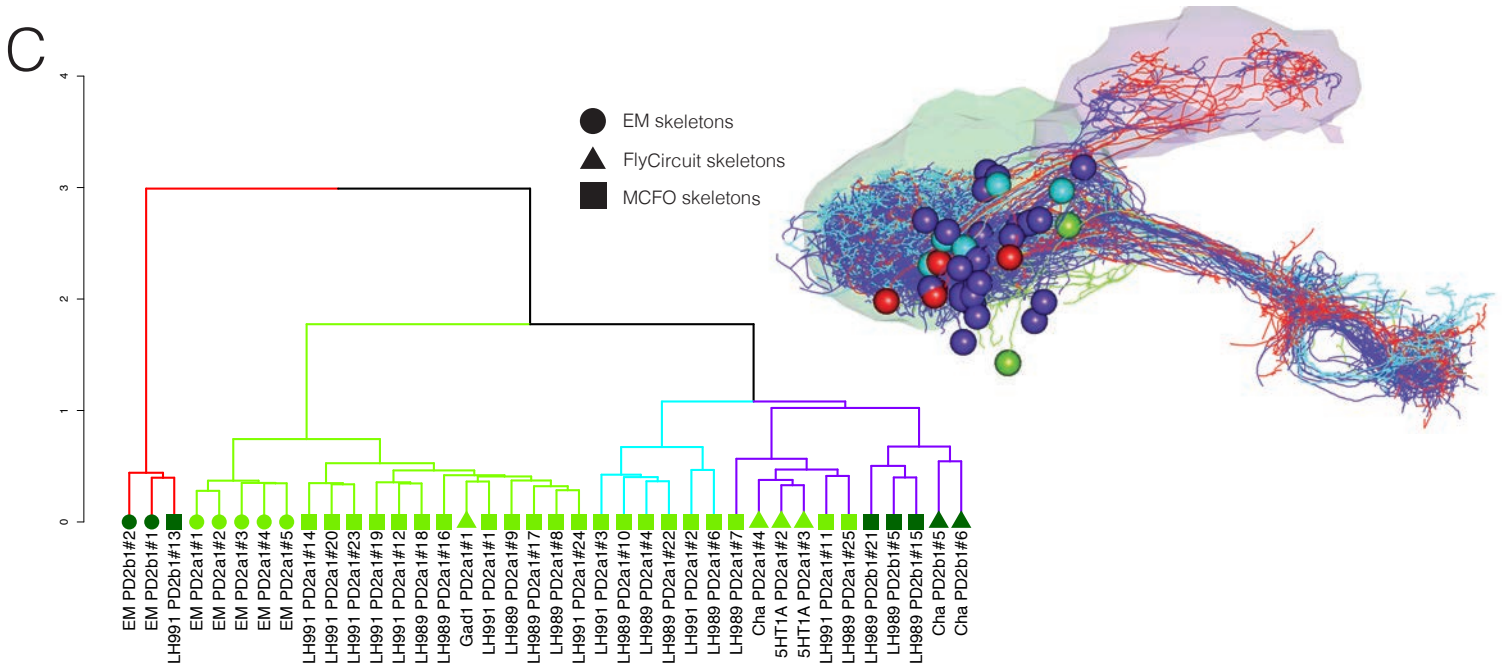


C'

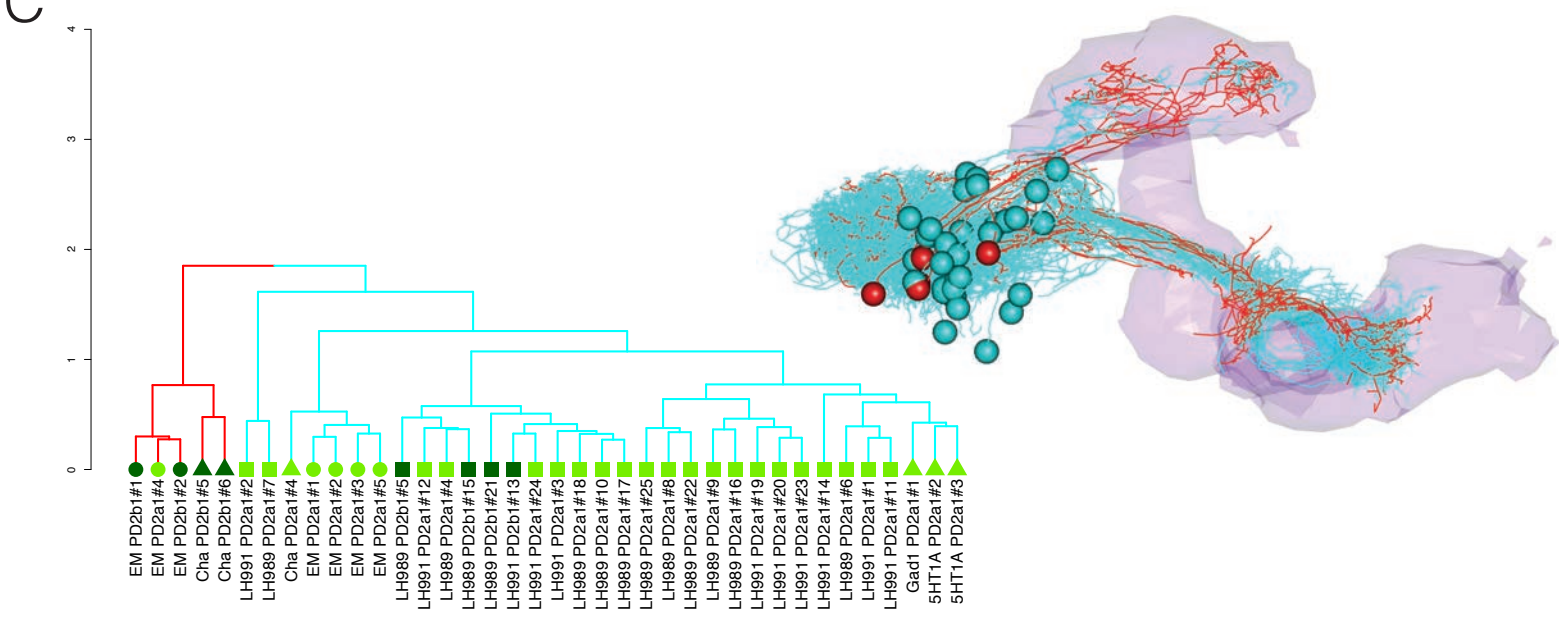
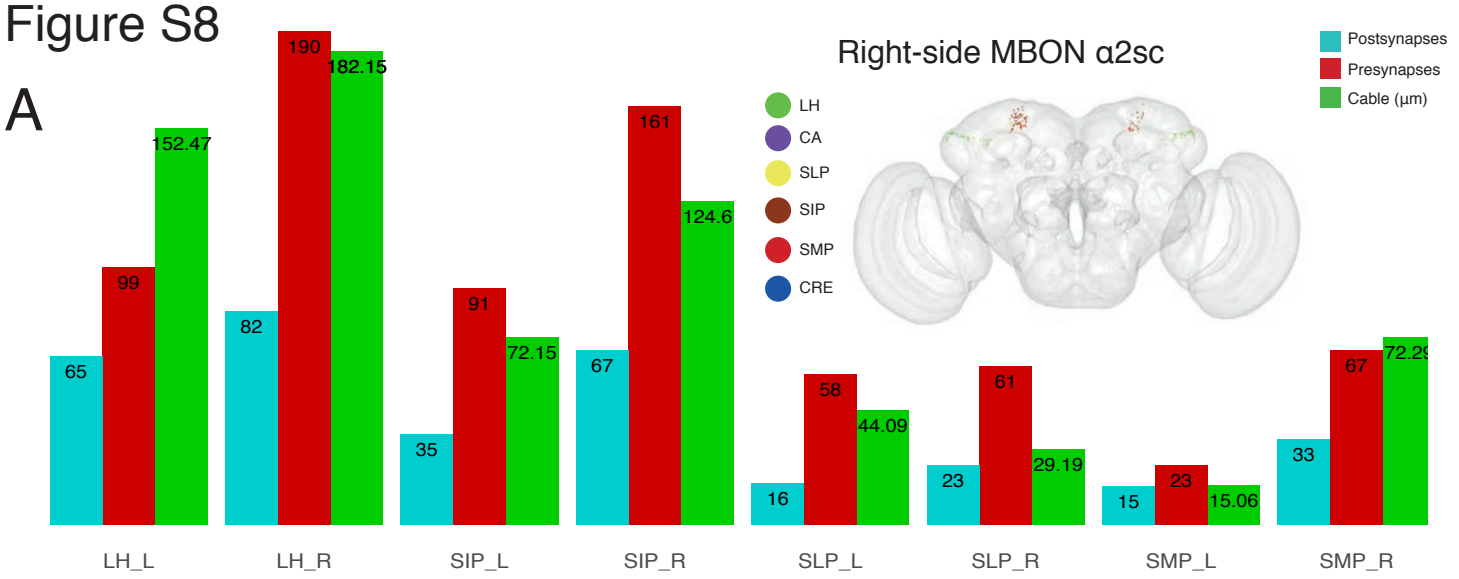
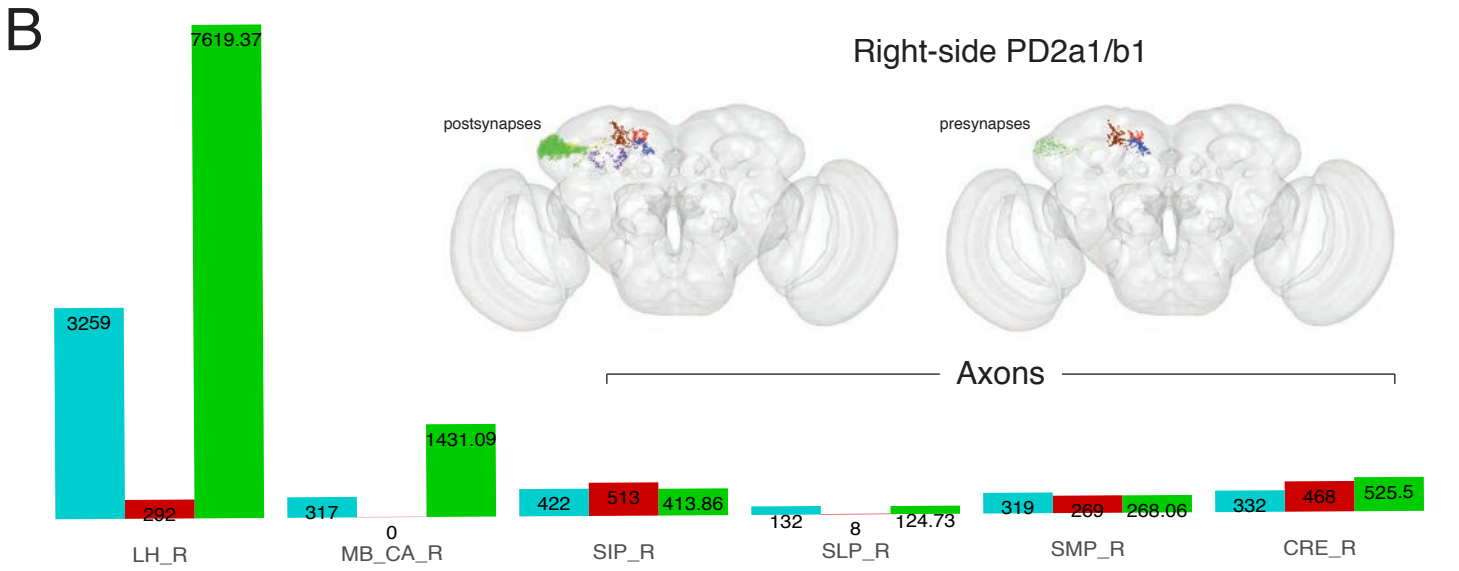
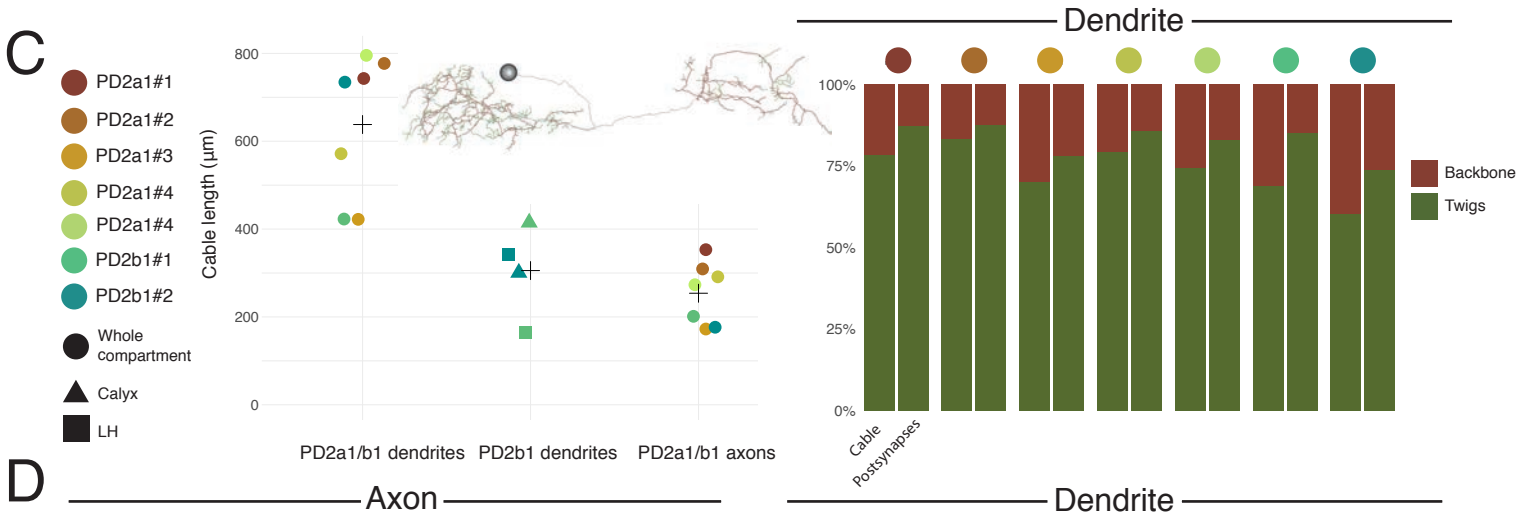


Figure S8


A
Right-side MBON α2sc
Postsynapses
Presynapses
Cable (μm)
LH
CA
SLP
SIP
SMP
CRE
65
99
152.47
82
190
182.15
35
91
72.15
67
161
124.6
16
58
44.09
23
61
29.19
15
23
15.06
33
67
72.29
LH_L
LH_R
SIP_L
SIP_R
SLP_L
SLP_R
SMP_L
SMP_R


B
Right-side PD2a1/b1
postsynapses
presynapses
Axons
3259
292
7619.37
317
0
1431.09
422
513
413.86
132
8
124.73
319
269
268.06
332
468
525.5
LH_R
MB_CA_R
SIP_R
SLP_R
SMP_R
CRE_R


C
PD2a1#1
PD2a1#2
PD2a1#3
PD2a1#4
PD2a1#4
PD2b1#1
PD2b1#2
Whole compartment
Calyx
LH
Cable length (μm)
800
600
400
200
0
PD2a1/b1 dendrites
PD2b1 dendrites
PD2a1/b1 axons
Dendrite
100%
75%
50%
25%
0%
Backbone
Twigs
Cable
Postsynapses

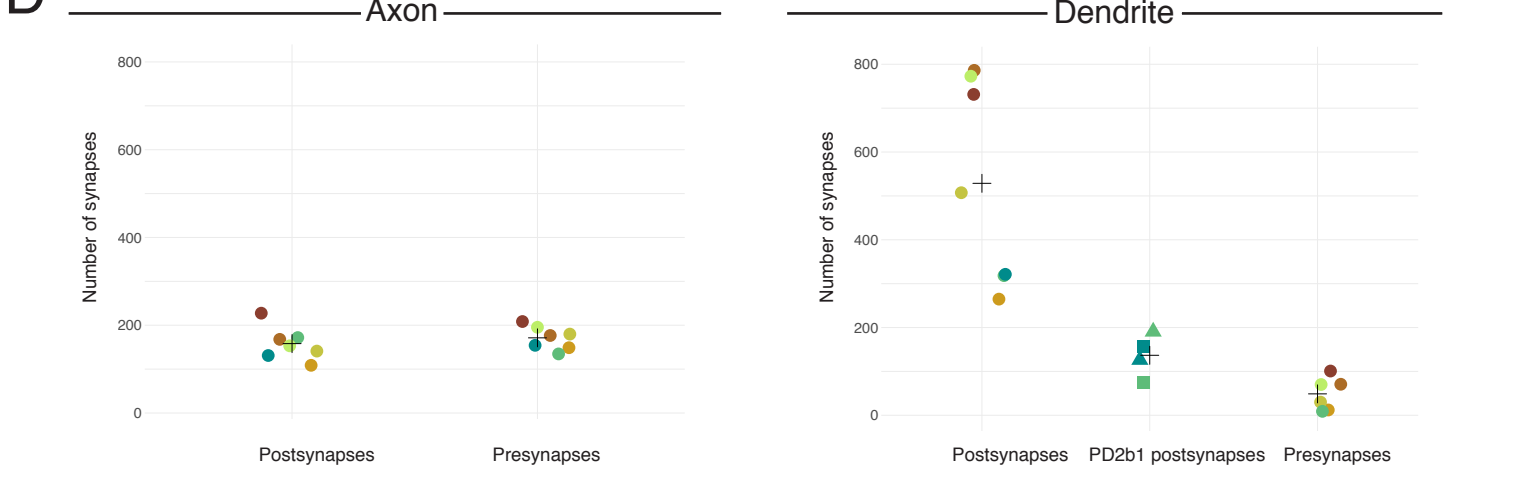

D
Axon
Number of synapses
800
600
400
200
0
Postsynapses
Presynapses
Dendrite
Number of synapses
800
600
400
200
0
Postsynapses
PD2b1 postsynapses
Presynapses

Figure S9

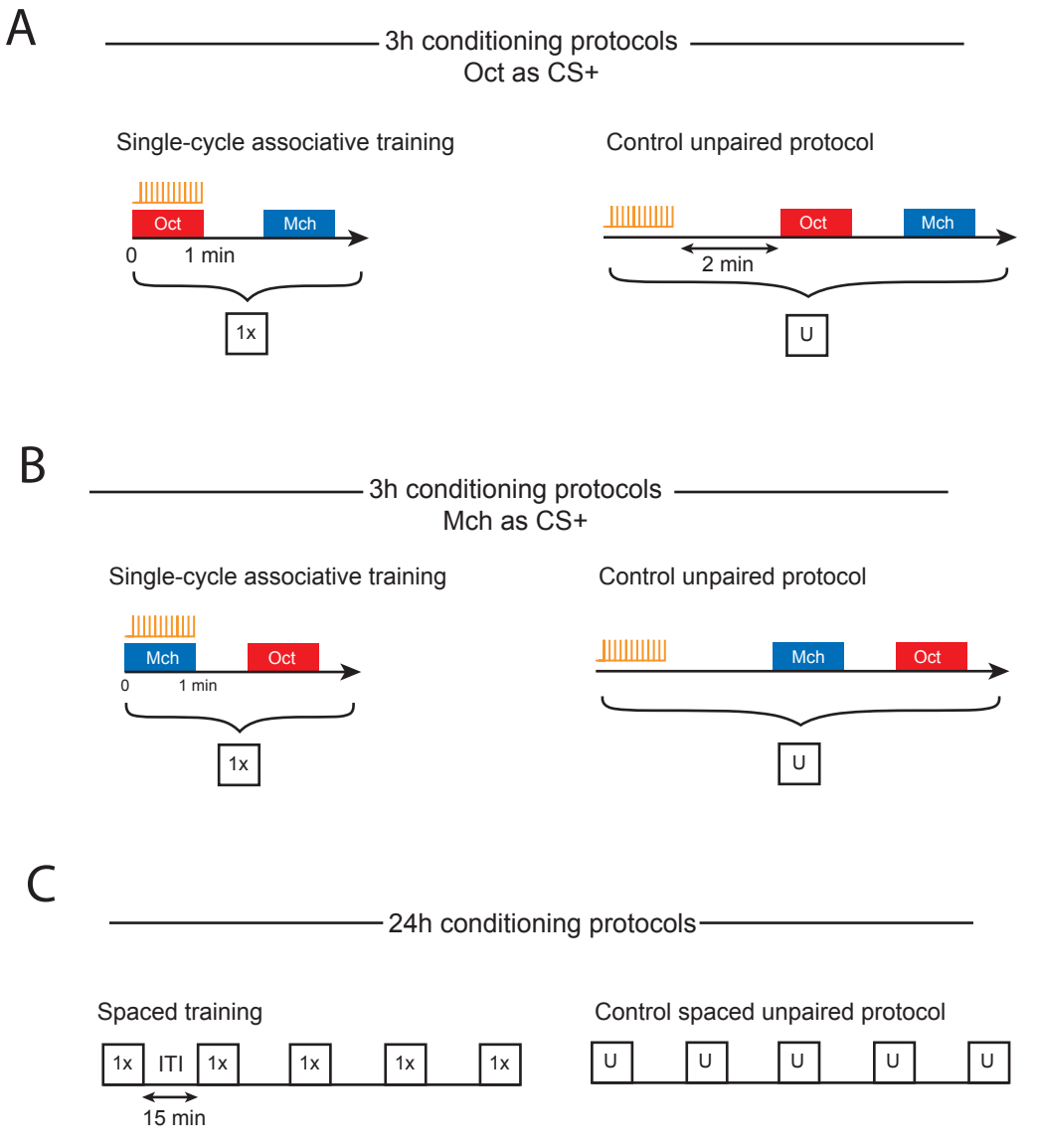
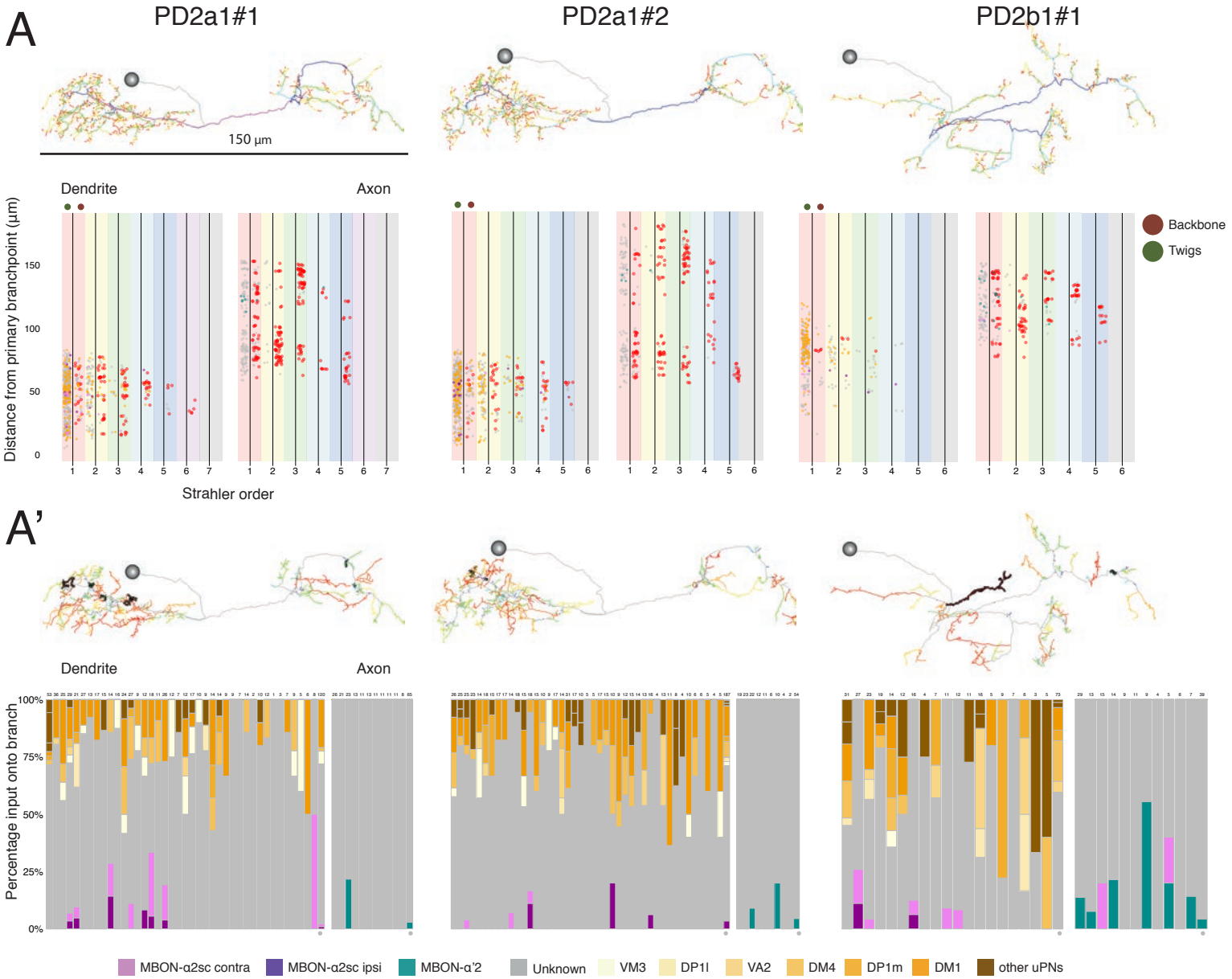
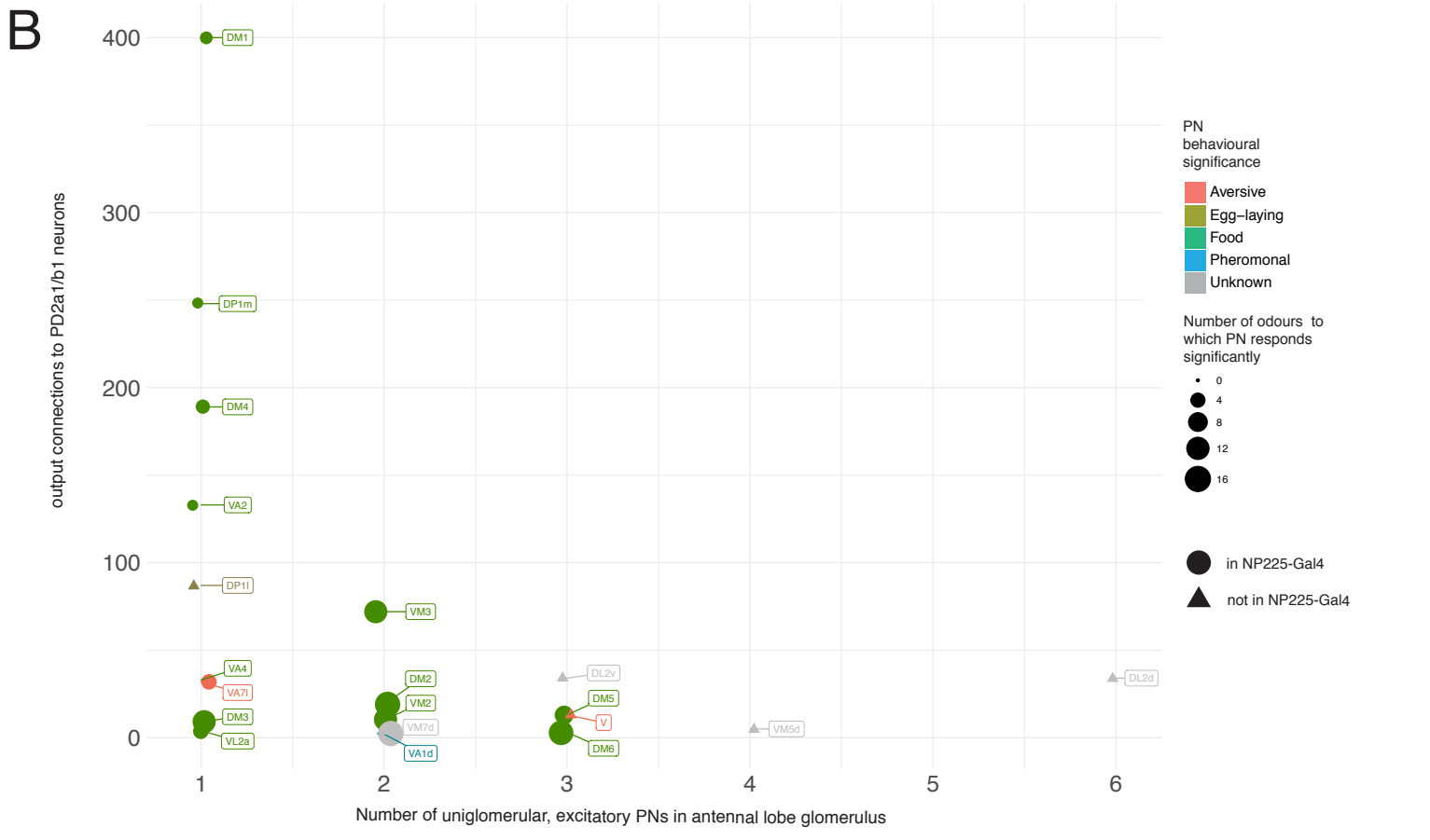

A
3h conditioning protocols
Oct as CS+
Single-cycle associative training
Oct
Mch
0
1 min
1x
Control unpaired protocol
Oct
Mch
2 min
U
B
3h conditioning protocols
Mch as CS+
Single-cycle associative training
Mch
Oct
0
1 min
1x
Control unpaired protocol
Mch
Oct
U
C
24h conditioning protocols
Spaced training
1x
ITI
1x
1x
1x
1x
15 min
Control spaced unpaired protocol
U
U
U
U
U

Figure S10


A
PD2a1#1
PD2a1#2
PD2b1#1
150 µm
Dendrite
Axon
Distance from primary branchpoint (µm)
Strahler order
Backbone
Twigs
A'
Dendrite
Axon
Percentage input onto branch
MBON-α2sc contra
MBON-α2sc ipsi
MBON-α'2
Unknown
VM3
DP1l
VA2
DM4
DP1m
DM1
other uPNs


B
output connections to PD2a1/b1 neurons
Number of uniglomerular, excitatory PNs in antennal lobe glomerulus
PN behavioural significance
Aversive
Egg–laying
Food
Pheromonal
Unknown
Number of odours to which PN responds significantly
in NP225-Gal4
not in NP225-Gal4