JNeurosci
THE JOURNAL OF NEUROSCIENCE

*Research Articles: Systems/Circuits*

# Aversive Training Induces Both Pre- and Postsynaptic Suppression in *Drosophila*

https://doi.org/10.1523/JNEUROSCI.1420-19.2019

**Cite as:** J. Neurosci 2019; 10.1523/JNEUROSCI.1420-19.2019

Received: 17 June 2019
Revised: 18 September 2019
Accepted: 22 September 2019

# Aversive Training Induces Both Pre- and Postsynaptic Suppression in *Drosophila*

Xiaofan Zhang[1], Nathaniel C. Noyes[1], Jianzhi Zeng[2], Yulong Li[2] and Ronald L. Davis[1]*
[1]Department of Neuroscience, The Scripps Research Institute Florida, Jupiter, FL 33458 USA

[2]State Key Laboratory of Membrane Biology, Peking University School of Life Sciences, PKU-IDG/McGovern Institute for Brain Research, Peking-Tsinghua Center for Life Sciences, Chinese Institute for Brain Research, Beijing 100871 China

Abbreviated Title: Cellular memory trace formation across the synapse

*Corresponding Author and Lead Contact address:
Ronald L. Davis
Department of Neuroscience
Scripps Research Institute Florida
Jupiter, Florida 33458
Tel: 561-228-3463
Fax: 561-228-3049
email: rdavis@scripps.edu

Number of pages: 35
Number of figures and multimedia: 6 main figures
Number of words for abstract, introduction, and discussion: 221 (abstract), 622 (introduction) and 837 (discussion)

**Acknowledgments**
This work was supported by NIH grant 1R35NS097224 to R.L.D. We thank the Fly Light Research Team at Janelia Research for providing *gal4* lines and the *20XUAS-shibire$^{ts}$* line.

**Conflict of Interest**
The authors declare no competing financial interests.

**Author Contributions**
X.Z., N.C.N. and R.L.D. planned all of the experiments. X.Z. performed the olfactory memory experiments and *in vivo* imaging experiments with GCaMP6f. He analyzed the data and wrote the initial draft of the manuscript along with N.C.N. X.Z. and N.C.N. performed the *in vivo* imaging experiments with GACh. J.Z. and Y.L. constructed the GACh reporter and transgenic flies. R.L.D. oversaw the execution of the project, contributed to the interpretation of the data and edited the manuscript.

**Abstract**

The α'β' subtype of *Drosophila* mushroom body neurons (MBn) is required for memory acquisition, consolidation and early memory retrieval after aversive olfactory conditioning. However, *in vivo* functional imaging studies have failed to detect an early forming memory trace in these neurons as reflected by an enhanced G-CaMP signal in response to presentation of the learned odor. Moreover, whether cellular memory traces form early after conditioning in the mushroom body output neurons (MBOn) downstream of the α'β' MBn remains unknown. Here, we show that aversive olfactory conditioning suppresses the calcium responses to the learned odor in both α'3 and α'2 axon segments of α'β' MBn and in the dendrites of α'3 MBOn immediately after conditioning using female flies. Notably, the cellular memory traces in both α'3 MBn and α'3 MBOn are short-lived and persist for less than 30 min. The suppressed response in α'3 MBn is accompanied by a reduction of acetylcholine (ACh) release, suggesting that the memory trace in postsynaptic α'3 MBOn may simply reflect the suppression in presynaptic α'3 MBn. Furthermore, we show that the α'3 MBn memory trace does not occur from the inhibition of GABAergic neurons via $GABA_A$ receptor activation. Since activation of the α'3 MBOn drives approach behavior of adult flies, our results demonstrate that aversive conditioning promotes avoidance behavior through suppression of the α'3 MBn-MBOn circuit.

**Significance Statement**

*Drosophila* learn to avoid an odor if that odor is repeatedly paired with electric shock. Mushroom body neurons (MBn) are known to be major cell types that mediate this form of aversive conditioning. Here we show that aversive conditioning causes a reduced response to the conditioned odor in an axon branch of one subtype of the MBn for no more than 30 min after conditioning, and in the dendrites of post-synaptic, MB output neurons (MBOn). Since experimenter-induced activation of the MBOn induces approach behavior by the fly, our data support a model that aversive learning promotes avoidance by suppressing the MBn-MBOn synapses that normally promote attraction.

77 **Introduction**

78 Animals learn to avoid a neutral stimulus that is repeatedly coupled with an unpleasant
79 one. This type of learning, aversive associative learning, induces cellular memory traces
80 in engram cells in the brain and changes the representation of the neutral stimulus
81 (Davis, 2011; Tonegawa et al., 2015). In *Drosophila*, several memory traces detected
82 with the calcium indicator G-CaMP have been observed in the mushroom body (MB), a
83 brain region critical for olfactory learning and memory (Davis, 1993; Yu et al., 2006;
84 Wang et al., 2008; Davis, 2011; Cervantes-Sandoval et al., 2013). These memory
85 traces are detectable across discrete time periods extending from 30 min to several
86 days after training. However, memory traces that form immediately in the MB after
87 conditioning have not been detected with *in vivo* $Ca^{2+}$ imaging.
88
89 The MB is composed of ~2,000 intrinsic neurons in each hemisphere that integrates
90 olfactory cues received from antennal lobe projection neurons with aversive or
91 rewarding stimuli from two clusters (PPL1, PAM) of dopamine neurons (DAns)
92 (Schwaerzel et al., 2003; Mao and Davis, 2009; Claridge-Chang et al., 2009; Aso et al.,
93 2012; Burke et al., 2012; Liu et al., 2012). MBn are classified into three major subtypes -
94 α'β', αβ, and γ neurons, based on their birth order and projection patterns of their axons
95 in the brain (Crittenden et al., 1998; Lee et al., 1999). The axons of α'β' and αβ MBn
96 bifurcate and project within the vertical α'/α lobe and horizontal β'/β lobe neuropil, while
97 the axons of γ neurons project only within the horizontal γ lobe neuropil. Although each
98 of these MBn subtypes contributes to aversive olfactory memory, they do so at different
99 times after conditioning (Cervantes-Sandoval et al., 2013), with synaptic transmission

100 from the α'β' and γ MBn required for robust expression of early and intermediate-term
101 memory (immediate to 3 hr) and synaptic transmission from the αβ MBn having a more
102 pronounced role for memory expression after 3 hr. Importantly, although the α'β' MBn
103 are required for memory acquisition, consolidation and early memory retrieval (Krashes
104 et al., 2007; Cervantes-Sandoval et al., 2013), no immediate memory trace in α'β' MBn
105 has been detected using *in vivo* $Ca^{2+}$ imaging (Wang et al., 2008).
106
107 Five different types of MB output neurons (MBONs) tile the α'β' lobe with their dendritic
108 trees into five discrete compartments, matching the tiling by axon terminals from
109 presynaptic DAn (Mao and Davis, 2009; Aso et al., 2014a). Several of these MBONs are
110 required for aversive memory or appetitive memory expression, and intermediate-term
111 memory traces (~1-2 hr after conditioning) have been detected in some of these
112 neurons (Séjourné et al., 2011; Owald et al., 2015). However, early memory traces have
113 not been documented in these MBONs, and the relationship between such putative
114 traces and those in the presynaptic MBn is unexplored. Connectome studies revealed
115 that DAns make direct connection with MBONs (Eichler et al., 2017; Takemura et al.,
116 2017), opening the possibility that MBONs form traces independently of the MBn.
117
118 Here, we show that a cellular memory trace forms immediately after conditioning in the
119 MBn axons occupying the α'3 compartment and in the downstream α'3 MBON.
120 Functional $Ca^{2+}$ imaging reveals that aversive conditioning suppresses subsequent
121 responses to the learned odor in both the presynaptic α'3 compartment and the
122 postsynaptic α'3 MBON across a similar time period, suggestive of a causal relationship.

*In vivo* ACh imaging revealed that the suppressed $Ca^{2+}$ responses are accompanied by reduced ACh release in the α'3 compartment, supporting the model that the α'3 MBOn memory trace occurs from suppressed presynaptic activity. We also show that the conditioning-induced suppression in the α'3 compartment does not occur from increased inhibition through the Rdl $GABA_A$ receptor, indicating that mechanisms other than Rdl receptor activation are responsible for the suppression of activity.

**Materials and Methods**

*Fly Husbandry:* Fly stocks were cultured on standard food at room temperature. Crosses were kept at 25°C with 70% relative humidity and a 12 hr light, 12 hr dark cycle except for the *shibire$^{ts}$* experiments in which flies were raised on standard food at 23°C until training and testing (see below for details). Fly lines used in this study include *w$^{1118}$* (BDSC, 3605), *c305a-gal4* (Krashes et al., 2007), *MB027B split gal4* (Aso et al., 2014b), *20XUAS-IVS-GCaMP6f* (Chen et al., 2013), *20XUAS-shibire$^{ts}$* (Pfeiffer et al., 2012), *UAS-mCD::GFP* (BDSC, 32197), *UAS-Rdli8-10* (Liu et al., 2007), *UAS-GACh4.4* (Jing et al., 2018).

*Behavioral Experiments:* We used 2-6 day old flies of mixed gender for behavior experiments. Standard aversive olfactory associative conditioning was performed as described (Beck et al., 2000). About 50-60 flies were equilibrated in a room dimly lit with red light and with ~70% humidity for >30 min in fresh food vials. Then they were loaded into a training tube where they received the following stimuli in sequence: 30 sec air, 1 min of an odor (CS+) paired with 12 electric shock pulses at 90 V (1.25 sec each pulse;

12 pulses, 30 V in Figure 6B), 30 sec of air, 1 min of a second odor (CS-) without shocks, 30 sec air. We used 4-methylcyclohexanol (MCH) and 3-octanol (OCT) as odors for conditioning. To measure 3 min memory, flies were transferred into a T-maze where they were allowed to choose between two arms containing the two odors for 2 min. The performance index (PI) was calculated as (number of flies choosing the correct arm) - (number of flies choosing the incorrect arm) / (total number of flies). A PI = 1 means that all flies choose the correct arm, and a PI = 0 means that the flies choose equally between the two arms. To measure memory at a later time, flies were transferred back to food vial until testing. For *shibire*$^{ts}$ experiments, flies were trained in 23°C, then they were transferred to 30°C (for *shibire*$^{ts}$ activation) or 23°C (Ctrl) immediately after training until testing of 15 min memory at that temperature.

*In Vivo Imaging:* We used a customized chamber for *in vivo* training and imaging (Figure 1B) similar to that used in a previous report (Berry et al., 2018). Briefly, a single female fly was aspirated into a 200 µl pipette tip cut to allow only the head to be exposed. Females are used only because of their larger size. The proboscis of the fly was secured in the retracted position with myristic acid to reduce the movement of the brain during imaging. The fly was then located in a narrow slot cut from the lid of a 5 cm petri dish. A small piece of stainless steel foil with a hole in it was glued to a 5 cm petri dish and covered the slot and the fly to expose the head. The head was glued to the foil with UV glue; the antenna underneath the foil remained free of glue. A small optical window was cut in the head cuticle to expose the brain and the head was then covered with fresh saline (124 mM NaCl, 3 mM KCl, 20 mM MOPS, 1.5 mM $CaCl_2$, 4 mM

169 $MgCl_2 \cdot 6H_2O$, 5 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \cdot H_2O$, 10 mM trehalose, 7 mM sucrose, 10
170 mM glucose, pH 7.2). Then the petri dish with the fly was attached to a base platform
171 with magnets. To deliver electric shock pulses to the fly, a custom shock platform made
172 with shock grids used in standard aversive training was secured to the base platform so
173 that the fly legs were in contact but with sufficient room so that the fly could temporarily
174 break the contact.
175
176 The fly was positioned such that the α' lobe neuropil was in the vertical line of the
177 objective lens in order to facilitate distinguishing the α'3 and α'2 compartments. In this
178 position, the α'3 compartment appears first as a crescent-shaped object with successive
179 Z steps in the ventral direction. Moving ventrally, a donut-shaped ring comes into focus.
180 We defined the α'3 region as essentially half the distance between the dorsal tip of the α'
181 lobe neuropil and the ring. The ring disappears with deeper imaging, being replaced by
182 a triangular region of fluorescence. We defined the α'2 compartment for imaging
183 purposes as the triangular region. Further down, the fluorescence region becomes
184 circular and then eventually merges at the junction area with the β' lobe. These
185 definitions are consistent with our immunostaining results showing the MBn α'3/α'3
186 MBOn compartment with MB027B split *gal4* (Figure 4) and the MB compartments
187 illustrated in the literature (Aso et al., 2014b).
188
189 To deliver odors (MCH and OCT) to the fly, 100 ml/min air stream was diverted from
190 flowing through a 20 ml glass vial containing 10 ml mineral oil to flow through a 20 ml
191 glass vial containing 10 ml $1X10^{-3}$ dilution of odorant in mineral oil. This air stream was

192 then blended into a 1000 ml/min fresh air stream before reaching the fly through a ~3
193 mm glass pipette positioned approximately 1 cm from the fly's antenna. After dissection,
194 the flies were allowed to rest for 3 min under the microscope before odor exposure. For
195 GCaMP6f and GACh imaging, 5 sec of each odor was delivered to the flies with a 30
196 sec interstimulus interval to confirm that the fly was alive and responding. Then, each fly
197 was presented with 2 pulses of 5 sec odor A (MCH or OCT) and 2 pulses of 5 sec odor
198 B (OCT or MCH) ("Pre" response) in alternating order. Each odor stimulus was
199 separated by 30 sec of fresh air. In the paired training protocol, flies were trained using
200 a schedule identical to the protocol described for behavior (above) starting 5 min after
201 the Pre odor exposure. For the unpaired training protocol, 1 min of electric shock pulses
202 was presented 2 min after the Pre odor exposure (Figure 1C). Following training, the
203 flies were presented with another set of 2 pulses of 5 sec odor A and 2 pulses of 5 sec
204 odor B ("Post") identical to the "Pre" stimulation.
205
206 A Leica TCS SP8 confocal microscope with a 488 nm argon laser and 25X water-
207 immersion objective was used for imaging. Imaging began 1 min before the first 5 sec
208 odor exposure and ended 30 sec after last 5 sec odor exposure for both Pre and Post
209 recording. Images were collected with a HyD detector (495-545 nm) at 2 Hz at a
210 resolution of 256X256 pixels. The baseline fluorescence $F_0$ was calculated as the
211 average fluorescence across the 5 sec prior to each odor exposure. Odor responses
212 were calculated as the average fluorescence (normalized to $F_0$ for each frame) within 5
213 sec odor presentation. Responses to the two pulses of the same odor in Pre and Post
214 tests were then averaged as the Pre or Post response for each fly.

*Immunostaining:* For the immunostaining of GFP driven by *c305a-gal4* and *MB027B-gal4* (Figure 1A and Figure 4A), whole brains were isolated and processed as described (Jenett et al., 2012). The primary antibodies used were rabbit anti-GFP (1:1000, ThermoFisher, cat# A11122) and mouse anti-nc82 (1:50, DSHB, RRID: AB2314866). The secondary antibodies used were goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:800, ThermoFisher, cat# A11008) and goat anti-mouse IgG conjugated to Alexa Fluor 633 (1:500, ThermoFisher, cat# A21052). Images were collected using a 10X objective with a Leica TCS SP8 confocal microscope with 488 and 633 nm laser excitation. The step size was 1 μm and images were collected at a resolution of 515X512 pixels.

*Experimental Design and Statistical Analysis:* For behavioral experiments, a mixture of both male and female flies was used. For *in vivo* imaging, only female flies were used because of their larger size. Statistical analyses were performed using Prism 5 (Graphpad). All tests were two tailed and confidence levels were set at $\alpha = 0.05$. Non-parametric tests were used for imaging data, while parametric tests were used for olfactory memory scores (PI) as the values are normally distributed (Walkinshaw et al., 2015). Sample sizes and statistical tests used for each experiment are listed in the figure legends.

## Results

### Aversive olfactory conditioning transiently suppresses responses to the learned odor in the axons of α'β' MBn

Since prior behavioral studies demonstrated that the output of α'β' MBn is required during acquisition, consolidation and retrieval of memories at early times after conditioning, we searched for memory traces in the axons of these neurons to identify the plasticity that might underlie this requirement (Krashes et al., 2007; Wang et al., 2008; Tan et al., 2010; Cervantes-Sandoval et al., 2013). There are five compartments in α'β' lobe neuropil (Figure 1A), defined by the connections made with presynaptic modulatory DAn axons and the dendritic trees of postsynaptic MBOns (Mao and Davis, 2009; Aso et al., 2014a, 2014b). We first focused on the α'3 region. To detect possible changes in odor responses in the α'3 compartment due to associative conditioning, we employed *in vivo* functional imaging, training the flies under the confocal microscope and recording odor responses before (Pre) and after (Post) training (Figure 1B and 1C). We used a paired training protocol consisting of 12 pulses electric shock at 90V (US) presented simultaneously with a 1 min presentation of an odor (CS+) followed by a second unpaired odor (CS-) with a 30 sec inter-odor interval. To control for non-associative effects, we employed a protocol in which the CS+ and US were explicitly unpaired (Figure 1C). We validated our paired and unpaired training protocols using behavioral assays. The paired training protocol induced robust memory for the CS+ at both 3 min and 1 hr after training, whereas, the unpaired training protocol produced no memory at either timepoint (Figure 1D).

Prior to conditioning, the MBn axons in the α'3 compartment showed robust $Ca^{2+}$ responses to the two odors used as CS+ or CS-, 4-methylcyclohexanol (MCH) and 3-octanol (OCT), detected with the reporter GCaMP6f expressed using the α'β' MBn driver, c305a-*gal4* (Figure 1A and 1E). When we paired MCH (CS+) with 12 pulses electric shock followed by OCT without shock (CS-), we surprisingly observed a strong suppression of the $Ca^{2+}$ response in α'3 compartment to MCH but not OCT at 3 min after pairing (Figure 1E and 1F). This suppression was pairing specific since the suppression was not observed in unpaired group (Figure 1F). In addition, the suppression was not odor specific since we observed a significant suppression using OCT as CS+ and MCH as CS- (Figure 1G). A weaker training protocol with 6 pulses of 30V electric shock also induced significant suppression to CS+ in α'3 compartment (Figure 1H).

We then measured the duration of the suppressed response. For this, we imaged the responses in the α'3 compartment at 15, 30, and 45 min after paired and unpaired conditioning and compared them with pre-conditioning responses. We found that the suppression in the α'3 compartment persisted to 15 min but was absent at 30 and 45 min after conditioning (Figure 2A, MCH as CS+). We observed the same time course using OCT as CS+ (Figure 2B). We note that the post-training responses tended to increase with time when compared to pre-training responses for both odors and in both paired and unpaired groups (Figure 2A and 2B). The source of this drift is unknown, but the critical measure is the difference between the paired and unpaired conditions. Together, these results demonstrate that aversive olfactory conditioning induces a very

early cellular memory trace in the α'3 compartment that is registered as a suppressed response specifically to the trained odor. This memory trace is transient and persists for less than 30 min.

**The α'β' MBn, α'2 neuropil compartment also shows conditioning-induced suppression in CS+ odor responses**

We next asked if the conditioning-induced suppression is specific to the α'3 compartment or whether it generalizes to the vertical axons of the α'β' MBn. Like the axonal segments in the α'3 compartment, those in the α'2 compartment region (Figure 3A) are also innervated by DAn of the PPL1 cluster (Mao and Davis, 2009; Aso et al., 2014a). In addition, activation of α'3 MBOn and α'2 MBOn both drive approach behavior, shown by the fly's preference to the light used to stimulate these neurons with CsChrimson (Aso et al., 2014a). Indeed, we observed a significant suppression to the CS+ in the α'2 compartment at 3 min using the paired but not the unpaired conditioning protocol (Figure 3B and 3C, OCT as CS+). In addition, the suppression generalized to the other odor when used as CS+ (Figure 3D). The α'1 compartment may also show a similar memory trace, but this region was difficult to resolve. The combined results reveal that olfactory aversive conditioning suppresses $Ca^{2+}$ responses in the axon segments of the α'β' MBn residing in the α'3 and α'2 compartments.

**Aversive olfactory conditioning transiently suppresses responses to the learned odor in the dendrites of α'3 MBOn**

Because the dendrites of the α'3 MBOn innervate the α'3 compartment (Figure 4A), we wondered whether the suppressed responses of the axonal segments of α'β' MBn in the α'3 compartment would be transmitted to the α'3 MBOn. This was the simplest model, assuming direct innervation and transfer of information from MBn to MBOn. However, given the complexity of this neuropil, which contains the processes of α'β' MBn, PPL1 DAn, α'3 MBOn, octopaminergic/GABAergic anterior paired lateral (APL) neurons (Liu and Davis, 2009; Wu et al., 2013) and serotonergic/GABAergic dorsal paired medial (DPM) neurons (Lee et al., 2011; Haynes et al., 2015), any signal from α'β' MBn may easily be modulated in some way.

We expressed GCaMP6f specifically in the α'3 MBOn with the split *gal4* MB027B (Figure 4A) and monitored responses before and after conditioning. The standard pairing protocol of 12 pulses, 90V of electric shock along with 1 min of CS+ (MCH) induced a strong suppression of α'3 MBOn responses to both the CS+ and CS- at 3 min after conditioning (Figure 4B). This suppression was also observed with both odors in the unpaired groups. We wondered whether this strong, CS+/CS- and paired/unpaired-independent suppression might mask an associative conditioning-induced memory trace in α'3 MBOn. To explore this possibility, we employed weaker training protocols with reduced shock voltage and fewer pulses to minimize the hypothetical non-specific suppression.

We found that non-specific suppression was weak or undetectable using 6 shock pulses of 30V, and that pairing and CS+ (MCH) odor-specific suppression was revealed (Figure

4C and 4D). We did observe a slight but significant decrease in the responses to OCT (CS-) in both the paired and unpaired groups (Figure 4D), however, there was no significant difference in the magnitude of the decrease between the two groups (Figure 4F, 3 min time point). Again, the training-induced suppression in α'3 MBOn was specific to the CS+ odor, since we observed the same result when we used OCT as CS+ and MCH as CS- (Figure 4E). Together, these results show that olfactory aversive conditioning also suppresses the CS+ odor response properties of the postsynaptic α'3 MBOn immediately after conditioning.

Given that DAn and other neuron types innervate the α'3 compartment, we considered the possibility that training may induce distinct memory traces in presynaptic α'3 MBn axons and postsynaptic α'3 MBOn dendrites. To address this possibility, we studied the duration of α'3 MBOn suppression to the conditioned odor and compared it with the time course for suppression in presynaptic α'3 axons. Using the 6 pulses, 30V conditioning schedule, we found that the suppression in the α'3 MBOn dendrites persisted for at least 15 but not 30 min after conditioning using MCH as CS+ (Figure 4F). There was no significant difference between paired and unpaired group responses to the CS- at either time point (Figure 4F). We worried that a US consisting of 6 pulses at 30V may be too weak to induce suppression lasting at least 30 min after conditioning, so we performed an experiment using 12 pulses of 90V as the US. Using this stronger conditioning paradigm, we failed to observe significant suppression in the α'3 MBOn dendrites at 30 min after conditioning (Figure 4G). We conclude that the suppressed response to the

conditioned odor in the dendrites of the α'3 MBOn follows the same time course as that observed in the MBn axonal segments in the α'3 compartment.

**The output of the α'3 MBOn is required for normal memory performance early after conditioning**

Although prior studies revealed that α'3 MBOn activation drives approach behavior (Aso et al., 2014a), the requirement of these neurons in the early phases of aversive memory expression remains unknown. The memory trace described here and its time course suggests that the α'3 MBOn may be required for the formation of behavioral memory and/or its retrieval at early times after conditioning. We reasoned that if α'3 MBOn output is disrupted during memory retrieval, then the training-induced difference between the CS+ and CS- evoked α'3 MBOn output will be eliminated, which will result in an impairment of memory expression.

To probe this issue, we expressed the temperature sensitive *shibire*$^{ts}$ transgene (*shi*$^{ts}$) in α'3 MBOn with MB027B-*gal4* driver. When we blocked α'3 MBOn output immediately after aversive training by elevating the temperature for 15 min prior to and during testing, we found a significant impairment in memory expression (Figure 4H). These results indicate that the synaptic output of α'3 MBOn is required for the expression of early memory, and further suggest that the short-lived cellular memory trace which forms in the α'3 MBn axon segments and/or the α'3 MBOn, contributes to behavioral memory expression early after conditioning.

**Conditioning-induced suppression of calcium responses in the α'3 MBn axon segment is accompanied by reduced ACh release**

The results presented above indicate that olfactory aversive conditioning suppresses the $Ca^{2+}$ responses to CS+ odor in both the presynaptic α'3 MBn axon segments and in the postsynaptic α'3 MBOn dendrites. The similar time courses for the suppression in both the pre- and post-synaptic elements (Figure 2 and Figure 4F) is most consistent with the possibility that the postsynaptic memory trace is a reflection of the presynaptic memory trace. This model predicts that the reduced $Ca^{2+}$ responses to the conditioned odor observed in the presynaptic α'3 MBn axon segments translates into reduced neurotransmitter release and a corresponding reduction of $Ca^{2+}$ responses in the postsynaptic dendrites. To probe this possibility, we measured the release of ACh in α'3 compartment as detected by the dendrites of α'3 MBOn after aversive conditioning.

We expressed an ACh sensor GACh (Jing et al., 2018) in α'3 MBOn using MB027B-*gal4* and imaged the postsynaptic α'3 MBOn before and after conditioning with 6 pulses of 30V electric shock (Figure 5A). We found that pairing odor (MCH) with electric shock significantly suppressed the GACh signal using the paired but not the unpaired protocol when measured at 3 min after conditioning (Figure 5B). This suggests that paired conditioning leads to reduced ACh release in response to the CS+ odor in α'3 compartment. No suppression of GACh response to CS+ was observed at 30 min after conditioning (Figure 5C). Since the time window of reduced ACh release matches the time window of α'3 MBn/α'3 MBOn suppressed $Ca^{2+}$ responses, we conclude that the

suppressed $Ca^{2+}$ signal of the α'3 MBOn dendrites likely occurs by reduced ACh release from the α'3 MBn axon segments.

**Suppression in α'3 MBn axon segments is not due to increased GABAergic inhibition through the Rdl $GABA_A$ receptor**

Many different cellular or network models can explain the reduced ACh release by the α'β' MBn due to conditioning. One of the more attractive models envisions an increased inhibitory tone on the MBn due to conditioning. Part of the attraction of this inhibitory model stems from the broad innervation of the MB neuropil by the APL and DPM GABAergic neurons. To probe this model, we reduced GABAergic input by knocking down the Rdl ($GABA_A$) receptor in α'β' neurons using RNAi (Liu and Davis, 2009). When Rdl was reduced in α'β' neurons, we found that 3 min memory was enhanced using 12 pulses, 30V as the US (Figure 6B), consistent with previous reports (Liu et al., 2007; Liu and Davis, 2009). We failed to see this enhancement using 12 pulses, 90V as US (Figure 6A), probably due to a ceiling effect. We then measured the short-term cellular memory trace that forms in the α'β' MBn with and without reduced Rdl expression. When the receptor expression was reduced using RNAi knockdown, we failed to observe any impairment of the α'3 compartment memory trace with GCaMP6f (Figure 6C), indicating that memory trace formation is independent of Rdl-mediated GABAergic inhibition. Instead, the suppression to the CS+ odor in the knocked down group was perhaps more robust compared to the control (59% vs 44% reduction, Figure 6C compared to Figure 6D). This slight increase in suppression might underlie the enhanced 3 min memory when Rdl is reduced in α'β' neurons. These results argue

against a model for a substantial role of increased inhibition to explain the suppression to CS+ odors due to conditioning.

## Discussion

Here, we provide evidence for the existence of immediate cellular memory traces that form in at least two adjacent segments of the axons in the vertical lobe neuropil of the α'β' MBn and at least one (α'3 MBOn) of the corresponding output neurons. These memory traces, detected as decreased $Ca^{2+}$ responses to the CS+ odor immediately after conditioning when compared to pre-conditioning responses, and persisting for less than 30 min before the response properties return to the naïve state, are consistent with the fact that α'β' MBn are required for memory acquisition, consolidation and early memory retrieval (Krashes et al., 2007; Cervantes-Sandoval et al., 2013). Several other previously characterized early memory traces due to odor conditioning provide an interesting background to these newly discovered traces (Davis, 2011). The neurites of the DPM neurons innervating the vertical MB lobe neuropil exhibit an increased $Ca^{2+}$ response to the learned odor from ~30-70 min after conditioning (Yu et al., 2005; Cervantes-Sandoval and Davis, 2012). A memory trace forms in the antennal lobe, registered as the recruitment of new projection neuron activity in response to the learned odor, that lasts less than 10 min after conditioning (Yu et al., 2004). The activity of GABAergic APL neurons that synapse in the vertical lobe neuropil of the MBn is suppressed for a period of a few min after conditioning (Liu and Davis, 2009). And, *in vivo* functional imaging of the α'β' MBn axons revealed an early memory trace displayed as increased $Ca^{2+}$ influx by 30 min after conditioning that persists for at least an hour

(Wang et al., 2008; Tan et al., 2010; Cervantes-Sandoval and Davis, 2012). The action of these five memory traces, together along with other unknown traces, may provide the cellular modifications required for behavioral performance gains to be made across the first hour after conditioning. Memory traces in compartments other than α'3 and/or their MBOns may underlie the requirement of α'β' MBn for memory retrieval beyond the first hour (Séjourné et al., 2011; Cervantes-Sandoval et al., 2013; Owald et al., 2015).

However, the developmental trajectory of the memory traces forming in the α'β' MBn lobe is of additional interest. As indicated above, a cellular memory trace forms in these neurons by 30 min after conditioning that is manifested as an increased $Ca^{2+}$ response to the conditioned odor (Wang et al., 2008; Tan et al., 2010). The data presented here show that the α'β' MBn axons become suppressed across the first ~15 min after conditioning. The combined studies thus indicate that the CS+ odor response properties in the α'β' MBn axons are initially suppressed after conditioning but then become enhanced at later times. The time courses for the two cellular memory traces do not match exactly (0-15 min for the suppression and ~30-60 min for the increase) given our current data showing no detectable increase at 30 or 45 min, but this is easily explained by variation in the strength of conditioning or minor technical differences between the two studies. Thus, the most parsimonious conclusion is that the vertical axon compartments of the α'β' MBn initially exhibit a suppressed response to the CS+ followed by an increased response with the transition from suppression to enhancement occurring somewhere between ~30-45 min after conditioning. How this evolution in

462 response properties from negative to positive with time translates into behavioral
463 memory expression remains unclear.
464
465 The suppressed responses to the CS+ odor were found in both the axon segments of
466 the α'β' MBn and the dendrites of α'3 MBOn. Given that activation of α'3 MBOn drives
467 approach behavior (Aso et al., 2014a), our results are consistent with the model that
468 aversive conditioning promotes avoidance through suppressing the MBn-MBOn circuits
469 that signal positive valence, at least across the time that the MBOn responses are
470 suppressed. Notably, the memory traces in α'3 MBn and α'3 MBOn persisted for the
471 similar time, raising the question of whether the suppressed responses form
472 independently or whether the α'3 MBOn memory trace simply reflects the presynaptic
473 one. Our data support the model in which the suppressed α'β' MBn responses are
474 simply transmitted to the MBOn from reduced synaptic activity: the suppressed $Ca^{2+}$
475 response in α'3 MBn axon compartment is correlated with reduced ACh release and the
476 suppressed response in the α'3 MBOn dendrites. Our behavioral data (Figure 4H)
477 suggest that if the early cellular memory traces that form in the α'3 MBn-MBOn circuit
478 cannot be readout precisely, the expression of behavioral memory early after
479 conditioning becomes impaired. However, we cannot exclude the possibility that
480 memory traces can be formed independently in α'3 MBOn.
481
482 We formulated the hypothesis that the immediate suppression in α'3 MBn axons after
483 aversive conditioning might be due to enhanced GABAergic input to the α'3
484 compartment in an effort to delineate the underlying mechanism. However, we failed to

485 detect any impairment of the immediate suppression in α'3 axonal compartment when
486 we knocked down Rdl $GABA_A$ receptor in α'β' neurons. Thus, our data argue against
487 attributing the suppression in the α'3 compartment to GABAergic inhibition through
488 $GABA_A$ receptor.

489
490

## References


Aso Y et al. (2014a) Mushroom body output neurons encode valence and guide memory-based action selection in Drosophila. Elife 3.

Aso Y, Hattori D, Yu Y, Johnston RM, Iyer NA, Ngo T-T, Dionne H, Abbott L, Axel R, Tanimoto H, Rubin GM (2014b) The neuronal architecture of the mushroom body provides a logic for associative learning. Elife 3.

Aso Y, Herb A, Ogueta M, Siwanowicz I, Templier T, Friedrich AB, Ito K, Scholz H, Tanimoto H (2012) Three Dopamine pathways induce aversive odor memories with different stability. PLoS Genet 8.

Aso Y, Siwanowicz I, Bräcker L, Ito K, Kitamoto T, Tanimoto H (2010) Specific dopaminergic neurons for the formation of labile aversive memory. Curr Biol 20:1445–1451.

Beck CD, Schroeder B, Davis RL (2000) Learning performance of normal and mutant Drosophila after repeated conditioning trials with discrete stimuli. J Neurosci 20:2944–2953

Berry JA, Phan A, Davis RL (2018) Dopamine Neurons Mediate Learning and Forgetting through Bidirectional Modulation of a Memory Trace. Cell Rep 25:651-662.

Burke CJ, Huetteroth W, Owald D, Perisse E, Krashes MJ, Das G, Gohl D, Silies M, Certel S, Waddell S (2012) Layered reward signalling through octopamine and dopamine in Drosophila. Nature 492:433–437.

Cervantes-Sandoval I, Davis RL (2012) Distinct traces for appetitive versus aversive olfactory memories in DPM neurons of Drosophila. Curr Biol 22:1247–1252.

514 Cervantes-Sandoval I, Martin-Pena A, Berry JA, Davis RL (2013) System-Like
515 Consolidation of Olfactory Memories in Drosophila. J Neurosci 33:9846–9854.
516 Chen TW, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, Schreiter ER, Kerr
517 RA, Orger MB, Jayaraman V, Looger LL, Svoboda K, Kim DS (2013) Ultrasensitive
518 fluorescent proteins for imaging neuronal activity. Nature 499:295–300.
519 Claridge-Chang A, Roorda RD, Vrontou E, Sjulson L, Li H, Hirsh J, Miesenbock G (2009)
520 Writing memories with light-addressable reinforcement circuitry. Cell 139:405–415.
521 Crittenden JR, Skoulakis EM, Han KA, Kalderon D, Davis RL (1998) Tripartite
522 mushroom body architecture revealed by antigenic markers. Learn Mem 5:38–51.
523 Davis RL (1993) Mushroom bodies and drosophila learning. Neuron 11:1–14.
524 Davis RL (2011) Traces of Drosophila Memory. Neuron 70:8–19.
525 Eichler K, Li F, Litwin-Kumar A, Park Y, Andrade I, Schneider-Mizell CM, Saumweber T,
526 Huser A, Eschbach C, Gerber B, Fetter RD, Truman JW, Priebe CE, Abbott LF,
527 Thum AS, Zlatic M, Cardona A (2017) The complete connectome of a learning and
528 memory centre in an insect brain. Nature 548:175–182.
529 Haynes PR, Christmann BL, Griffith LC (2015) A single pair of neurons links sleep to
530 memory consolidation in Drosophila melanogaster. Elife 4.
531 Jenett A et al. (2012) A GAL4-Driver Line Resource for Drosophila Neurobiology. Cell
532 Rep 2:991–1001.
533 Jing M et al. (2018) A genetically encoded fluorescent acetylcholine indicator for in vitro
534 and in vivo studies. Nat Biotechnol 36:726–737.
535 Krashes MJ, Keene AC, Leung B, Armstrong JD, Waddell S (2007) Sequential Use of
536 Mushroom Body Neuron Subsets during Drosophila Odor Memory Processing.

Neuron 53:103–115.

Lee P-T, Lin H-W, Chang Y-H, Fu T-F, Dubnau J, Hirsh J, Lee T, Chiang A-S (2011) Serotonin-mushroom body circuit modulating the formation of anesthesia-resistant memory in Drosophila. Proc Natl Acad Sci U S A 108:13794–13799.

Lee T, Lee A, Luo L (1999) Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. Development 126:4065–4076.

Liu C, Plaşais PY, Yamagata N, Pfeiffer BD, Aso Y, Friedrich AB, Siwanowicz I, Rubin GM, Preat T, Tanimoto H (2012) A subset of dopamine neurons signals reward for odour memory in Drosophila. Nature 488:512–516.

Liu X, Davis RL (2009) The GABAergic anterior paired lateral neuron suppresses and is suppressed by olfactory learning. Nat Neurosci 12:53–59.

Liu X, Krause WC, Davis RL (2007) GABAA receptor RDL inhibits Drosophila olfactory associative learning. Neuron 56:1090–1102.

Mao Z, Davis RL (2009) Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity. Front Neural Circuits 3:5.

Owald D, Felsenberg J, Talbot CB, Das G, Perisse E, Huetteroth W, Waddell S (2015) Activity of defined mushroom body output neurons underlies learned olfactory behavior in Drosophila. Neuron 86:417–427.

Pfeiffer BD, Truman JW, Rubin GM (2012) Using translational enhancers to increase transgene expression in Drosophila. Proc Natl Acad Sci 109:6626–6631.

Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelin F, Birman S, Heisenberg M (2003)

560 Dopamine and octopamine differentiate between aversive and appetitive olfactory
561 memories in Drosophila. J Neurosci 23:10495–10502.
562 Séjourné J, Plaçais P-Y, Aso Y, Siwanowicz I, Trannoy S, Thoma V, Tedjakumala SR,
563 Rubin GM, Tchénio P, Ito K (2011) Mushroom body efferent neurons responsible
564 for aversive olfactory memory retrieval in Drosophila. Nat Neurosci 14:903–910.
565 Takemura S et al. (2017) A connectome of a learning and memory center in the adult
566 Drosophila brain. Elife 6.
567 Tan Y, Yu D, Pletting J, Davis RL (2010) Gilgamesh Is Required for rutabaga-
568 Independent Olfactory Learning in Drosophila. Neuron 67:810–820.
569 Tonegawa S, Liu X, Ramirez S, Redondo R (2015) Memory Engram Cells Have Come
570 of Age. Neuron 87:918–931.
571 Walkinshaw E, Gai Y, Farkas C, Richter D, Nicholas E, Keleman K, Davis RL (2015)
572 Identification of genes that promote or inhibit olfactory memory formation in
573 Drosophila. Genetics 199:1173–1182.
574 Wang Y, Mamiya A, Chiang A, Zhong Y (2008) Imaging of an early memory trace in the
575 Drosophila mushroom body. J Neurosci 28:4368–4376.
576 Wu CL, Shih MFM, Lee PT, Chiang AS (2013) An octopamine-mushroom body circuit
577 modulates the formation of anesthesia-resistant memory in drosophila. Curr Biol
578 23:2346–2354.
579 Yu D, Akalal D-BG, Davis RL (2006) Drosophila α/β Mushroom Body Neurons Form a
580 Branch-Specific Long-Term Cellular Memory Trace after Spaced Olfactory
581 Conditioning. Neuron 52:845–855.
582 Yu D, Keene AC, Srivatsan A, Waddell S, Davis RL (2005) Drosophila DPM neurons

583 form a delayed and branch-specific memory trace after olfactory classical
584 conditioning. Cell 123:945–957.
585 Yu D, Ponomarev A, Davis RL (2004) Altered representation of the spatial code for
586 odors after olfactory classical conditioning: memory trace formation by synaptic
587 recruitment. Neuron 42:437–449.
588
589
590

591 **Figure Legends**

592 **Figure 1. Pairing of Odor and Electric Shock Induces Suppression to CS+ Odors**
593 **in the α'3 Compartment**

594 (A) Left: schematic diagram of MB showing α'β' lobe neuropil and its five compartments
595 from a frontal perspective. The line across α'3 indicates the plane that was selected for
596 imaging. Middle: morphology of MB α'β' lobe neuropil visualized using c305a-*gal4* >
597 mCD8::GFP. Right: a representative *in vivo* image of GCaMP6f expression in the α'3
598 region driven by c305a-*gal4* (scale bar, 10 μm; brightness and contrast were adjusted
599 for better visualization).
600 (B) Schematic diagram of the *in vivo* training setup. The fly head is glued to a thin metal
601 plate. There is a small hole in the plate through which the head cuticle is dissected and
602 the brain can be imaged. The electric shock pulses are delivered to the fly through an
603 electric grid contacting the fly's legs. Odor is delivered to the fly via glass pipette (~3
604 mm diameter) whose tip is close to the antenna of the fly (arrows indicate odor flow
605 direction).
606 (C) Paired and unpaired training protocol. In paired training, 1 min of 12 pulses electric
607 shock and odor A (CS+) were presented simultaneously followed by odor B (CS-) that
608 was unpaired with shock. In unpaired training, 1 min of electric shock pulses was
609 presented 3 min before odor A onset. Prior to training, two pulses of each odor were
610 presented to the fly in alternating fashion for 5 sec each with a 30 sec interstimulus
611 interval (Pre). After training, an identical set of odor pulses were presented to the fly
612 (Post).

(D) Behavior performance of flies receiving paired and unpaired training protocols. Paired training induced robust memory performance at both 3 min and 1 hr after training. However, unpaired training induced no memory at 3 min or 1 hr after training. Mean ± SEM, ***$p < 0.0001$, ns, not significant, $p \geq 0.4976$, one sample t-test against a theoretical mean of "0", $n = 6$.

(E) Pseudocolored peak responses of α'3 axon segments to CS+ and CS- before (Pre) and 3 min after (Post) paired training.

(F) Left: top, time course of GCaMP6f response in α'3 to CS+ (MCH) during 5 sec odor presentation before (Pre, blue) and 3 min after (Post, red) paired or unpaired training. Traces show the average response (±SEM) across all flies tested. Bottom: mean odor-evoked Pre and Post responses during the 5 sec odor presentation. Right: responses to the CS- (OCT). Mean ± SEM; ***$p = 0.0003$; ns, not significant, $p > 0.9999$; repeated-measures two-way ANOVA with Bonferroni post hoc tests, $n = 8$.

(G) Left: top, time course of GCaMP6f response in α'3 to OCT (CS+) during a 5 sec odor presentation before (Pre, blue) and 3 min after (Post, red) paired or unpaired training. Traces show the average response (±SEM) across all flies tested. Bottom: mean odor-evoked Pre and Post responses during 5 sec odor presentation. Right: responses to the CS- (MCH). Mean ± SEM; **$p = 0.0022$; ns, not significant, $p \geq 0.4230$; repeated-measures two-way ANOVA with Bonferroni post hoc tests, $n = 8$.

(H) Top: mean odor-evoked Pre and Post responses during 5 sec odor presentation to MCH (CS+) with 6 pulses of 30V electric shock training protocol. Bottom: responses to the CS- (OCT). Mean ± SEM, **$p = 0.0077$; ns, not significant, $p \geq 0.6267$; repeated-measures two-way ANOVA with Bonferroni post hoc tests, $n = 6$.

**Figure 2. Conditioning-induced Suppression to CS+ in the α'3 Compartment Persists less than 30 min**

(A) Change of odor response in α'3 axon segments between Post and Pre odor stimuli at 3, 15, 30, and 45 min after paired or unpaired training with MCH as CS+ and OCT as CS-. The change of odor response within each fly was calculated as (Post-Pre)/Pre. The paired training-induced suppression of the CS+ persisted for at least 15 min and became non-significant at 30 and 45 min. No significant difference was observed in CS- between paired and unpaired groups at any time point. Mean ± SEM; ***$p$ = 0.0002, **$p$ = 0.0047; ns, not significant, $p \geq 0.2345$; Mann-Whitney U test, $n$ = 8.

(B) Change of odor response in the α'3 compartment between Post and Pre at 3, 15, 30, and 45 min after paired or unpaired training with OCT as CS+ and MCH as CS-. The paired training-induced suppression of the CS+ persisted for at least 15 min and became non-significant at 30 and 45 min. No significant difference was observed in CS- between paired and unpaired groups at any time point. Mean ± SEM; ***$p$ =0.0006, *$p$ = 0.0379; ns, not significant, $p \geq 0.1304$; Mann-Whitney U test, $n$ = 8.

**Figure 3. Pairing of Odor and Electric Shock Induces Suppression to CS+ Odors in the α'2 Compartment**

(A) Left: schematic diagram of MB showing α'β' lobe neuropil and its five compartments. The line across α'2 indicates the plane that was selected for imaging. Middle: morphology of MB α'β' lobe visualized using c305a-*gal4* > mCD8::GFP. Right: a representative *in vivo* image of GCaMP6f expression in the α'2 region driven by c305a-*gal4* (scale bar, 10 μm; brightness and contrast were adjusted for better visualization).

(B) Pseudocolored peak responses of α'2 to CS+ and CS- before (Pre) and 3 min after (Post) paired training.

(C) Left: top, time course of GCaMP6f response in α'2 to CS+ (OCT) during 5 sec odor presentation before (Pre, blue) and 3 min after (Post, red) paired or unpaired training. Traces show the average response (±SEM) across all flies tested. Bottom: mean odor-evoked Pre and Post responses during 5 sec odor presentation. Right: responses to the CS- (MCH). Mean ± SEM; $^{**}p = 0.0085$; ns, not significant, $p \geq 0.3129$; repeated-measures two-way ANOVA with Bonferroni post hoc tests, $n = 9$.

(D) Left: top, time course of GCaMP6f response in α'2 to MCH (CS+) during a 5 sec odor presentation before (Pre, blue) and 3 min after (Post, red) paired or unpaired training. Traces show the average response (±SEM) across all flies tested. Bottom: mean odor-evoked Pre and Post responses during 5 sec odor presentation. Right: responses to the CS- (OCT). Mean ± SEM; $^{*}p = 0.0192$; ns, not significant, $p > 0.9999$; repeated-measures two-way ANOVA with Bonferroni post hoc tests, $n = 7$.

**Figure 4. Pairing of Odor and Electric Shock Induces Suppression in α'3 MBOn**

(A) Morphology of postsynaptic MBOn that innervates α'3 compartment visualized using MB027B split *gal4* > mCD8::GFP. The line across α'3 MBOn indicates the plane that was selected for imaging.

(B) Left: mean odor-evoked GCaMP6f responses in α'3 MBOn to MCH (CS+) during 5 sec odor presentation before (Pre, blue) and 3 min after (Post, red) paired or unpaired training with 12 pulses of 90V electric shock. Right: responses to OCT (CS-). Mean ±

681 SEM; ***$p$ = 0.0008, **$p \leq 0.0021$; repeated-measures two-way ANOVA with Bonferroni
682 post hoc tests, $n$ = 7.
683 (C) Pseudocolored peak responses of α'3 MBOn to CS+ (MCH) and CS- (OCT) before
684 (Pre) and 3 min after (Post) paired training with 6 pulses of 30V electric shock.
685 (D) Left: top, time course of GCaMP6f response in α'3 MBOn to CS+ (MCH) during 5
686 sec odor presentation before (Pre, blue) and 3 min after (Post, red) paired or unpaired
687 training with 6 pulses of 30V electric shock. Traces show the average response (±SEM)
688 across all flies tested. Bottom: Mean odor-evoked Pre and Post responses during the 5
689 sec odor presentation. Right: responses to the CS- (OCT). Mean ± SEM; ***$p$ = 0.0009,
690 *$p \leq 0.0119$; ns, not significant, $p$ = 0.2951; repeated-measures two-way ANOVA with
691 Bonferroni post hoc tests, $n$ = 6.
692 (E) Left: mean odor-evoked GCaMP6f responses in α'3 MBOn to OCT (CS+) during 5
693 sec odor presentation before (Pre, blue) and 3 min after (Post, red) paired or unpaired
694 training with 6 pulses of 30V electric shock. Right: responses to MCH (CS-). Mean ±
695 SEM; **$p$ = 0.0029; ns, not significant, $p \geq 0.1236$; repeated-measures two-way ANOVA
696 with Bonferroni post hoc tests, $n$ = 8.
697 (F) Change of odor response in α'3 MBOn between Post and Pre at 3, 15, and 30 min
698 after paired or unpaired training (CS+:MCH, CS-:OCT). The paired training-induced
699 suppression of CS+ persisted for at least 15 min and became non-significant at 30 min.
700 No significant difference was observed in CS- between paired and unpaired groups at
701 any time point. Mean ± SEM; **$p$ = 0.0087, *$p$ = 0.0411; ns, not significant, $p \geq 0.3095$;
702 Mann-Whitney U test, $n$ = 6.

(G) Mean odor-evoked GCaMP6f response at 30 min after training in α'3 MBOn during 5 sec odor presentation with 12 pulses of 90V electric shock (CS+:MCH, CS-:OCT). Mean ± SEM; ns, not significant, $p \geq 0.1372$; repeated-measures two-way ANOVA with Bonferroni post hoc tests, $n = 7$.

(H) Blocking synaptic output of α'3 MBOn immediately after training through testing impaired 15 min memory. Flies were trained at 23°C, transferred to 30°C immediately after training for 15 min, and tested at 30°C. Mean ± SEM; $^*p = 0.0186$; ns, not significant, $p > 0.9999$; two-way ANOVA with Bonferroni post hoc tests, $n = 6$.

**Figure 5. Pairing of Odor and Electric Shock Reduces ACh Release in the α'3 Compartment**

(A) Morphology of postsynaptic MBOn that innervates α'3 compartment visualized using MB027B split *gal4* > mCD8::GFP. The line across α'3 MBOn indicates the plane that was selected for imaging.

(B) Left: top, time course of GACh (ACh sensor) response in the dendrites of α'3 MBOn to CS+ (MCH) during 5 sec odor presentation before (Pre, blue) and 3 min after (Post, red) paired or unpaired training with 6 pulses of 30V electric shock. Traces show the average response (±SEM) across all flies tested. Bottom: mean odor-evoked Pre and Post responses during the 5 sec odor presentation. Right: responses to the CS- (OCT). Mean ± SEM; $^*p = 0.0457$; ns, not significant, $p > 0.9999$ for MCH in unpaired group, $p = 0.3003$ for OCT in paired group and $p = 0.0856$ for OCT in unpaired group; repeated-measures two-way ANOVA with Bonferroni post hoc tests, $n = 6$.

(C) Mean odor-evoked GACh response at 30 min after training in the dendrites of α'3 MBOn during 5 sec odor presentation with 6 pulses of 30V electric shock (CS+:MCH, CS-:OCT). Mean ± SEM; ns, not significant, $p \geq 0.6660$; repeated-measures two-way ANOVA with Bonferroni post hoc tests, $n = 6$.

**Figure 6. Suppression in α'3 is not Induced by GABAergic Inhibition Through Rdl $GABA_A$ Receptor**

(A) No change in 3 min memory with 12 pulses of 90V electric shock when Rdl ($GABA_A$ receptor) was knocked down in α'β' MBn. Mean ± SEM; ns, not significant; one-way ANOVA with Tukey's post hoc tests, $n = 6$.

(B) There was significant enhancement of 3 min memory expression with 12 pulses of 30V electric shock when Rdl was knocked down in α'β' MBn. Mean ± SEM; $*p \leq 0.0391$; one-way ANOVA with Tukey's post hoc tests, $n = 6$.

(C) Knocking down Rdl did not impair the odor suppression to the CS+ in the α'3 compartment. Left: top, time course of GCaMP6f response in the α'3 compartment to the CS+ (MCH) during a 5 sec odor presentation before (Pre, blue) and 3 min after (Post, red) paired or unpaired training. Traces show the average response (±SEM) across all flies tested. Bottom: mean odor-evoked Pre and Post responses during 5 sec odor presentation. Right: responses to the CS- (OCT). Mean ± SEM; $***p = 0.0009$; ns, not significant, $p \geq 0.1515$; repeated-measures two-way ANOVA with Bonferroni post hoc tests, $n = 8$.

(D) Control for Rdl knockdown in α'β' MBn. Left: top, time course of GCaMP6f response in the α'3 compartment to the CS+ (MCH) during 5 sec odor presentation before (Pre,

748 blue) and 3 min after (Post, red) paired or unpaired training. Traces show the average
749 response (±SEM) across all flies tested. Bottom: Mean odor-evoked Pre and Post
750 responses during 5 sec odor presentation. Right: responses to the CS- (OCT). Mean ±
751 SEM; ***$p$ = 0.0006; ns, not significant, $p \geq 0.5049$; repeated-measures two-way
752 ANOVA with Bonferroni post hoc tests, $n$ = 6.

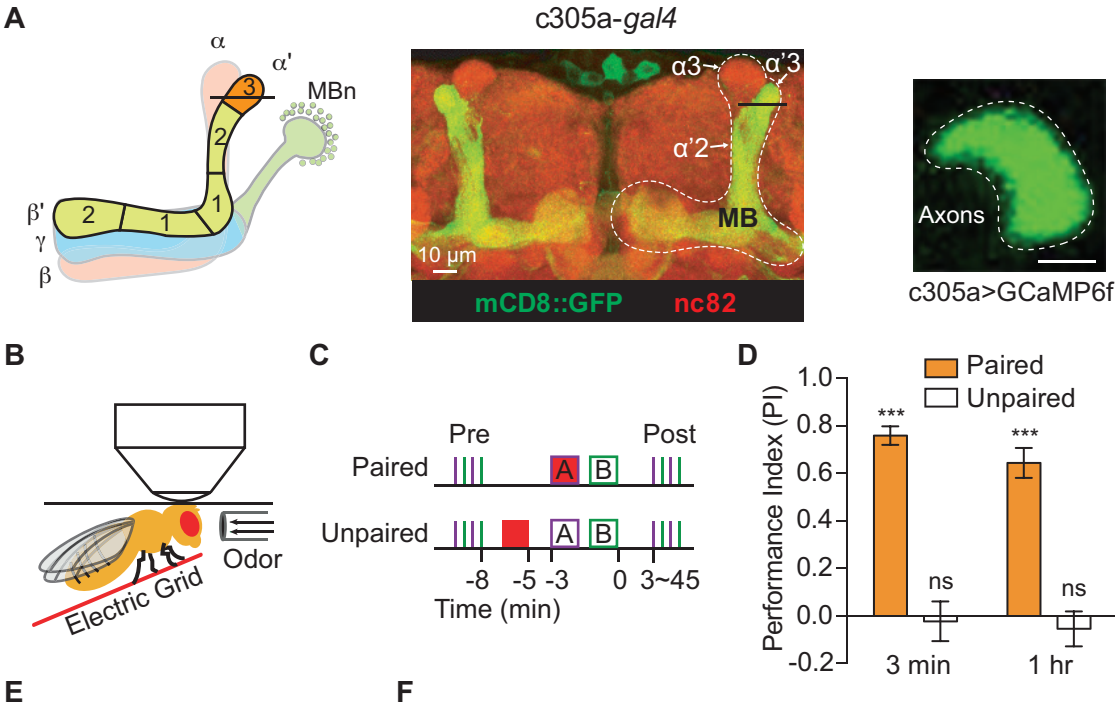


Figure 1

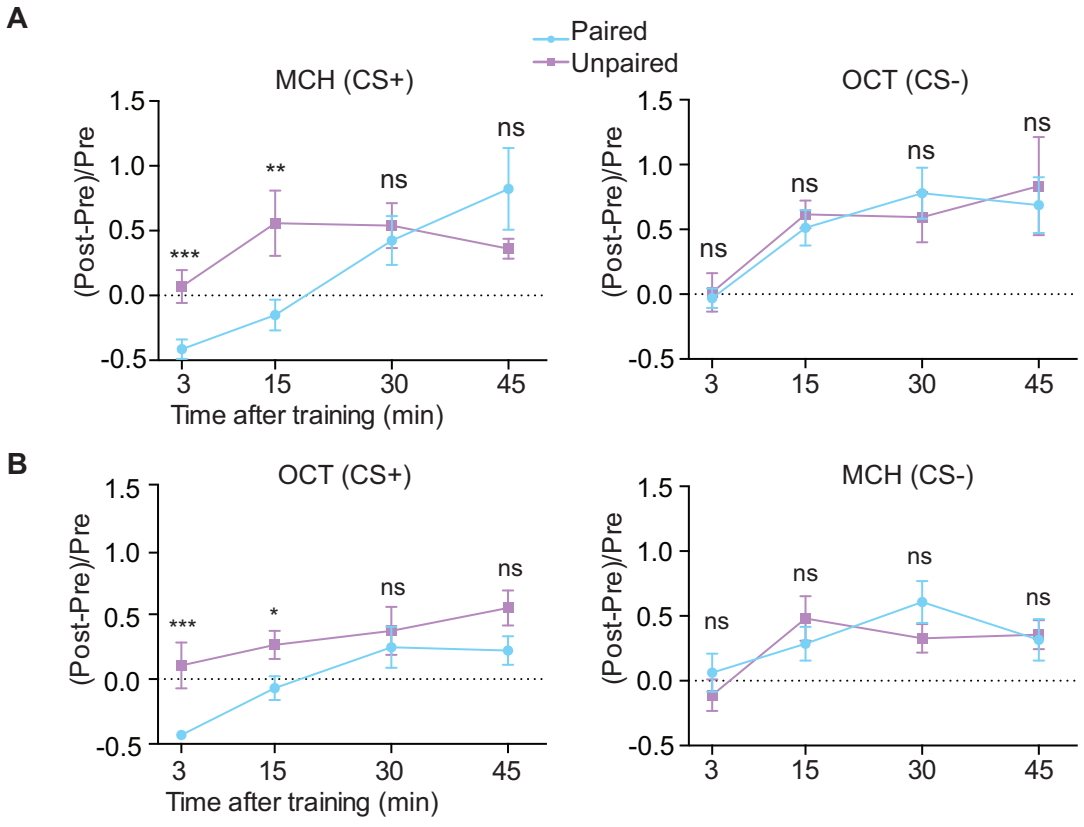


Figure 2

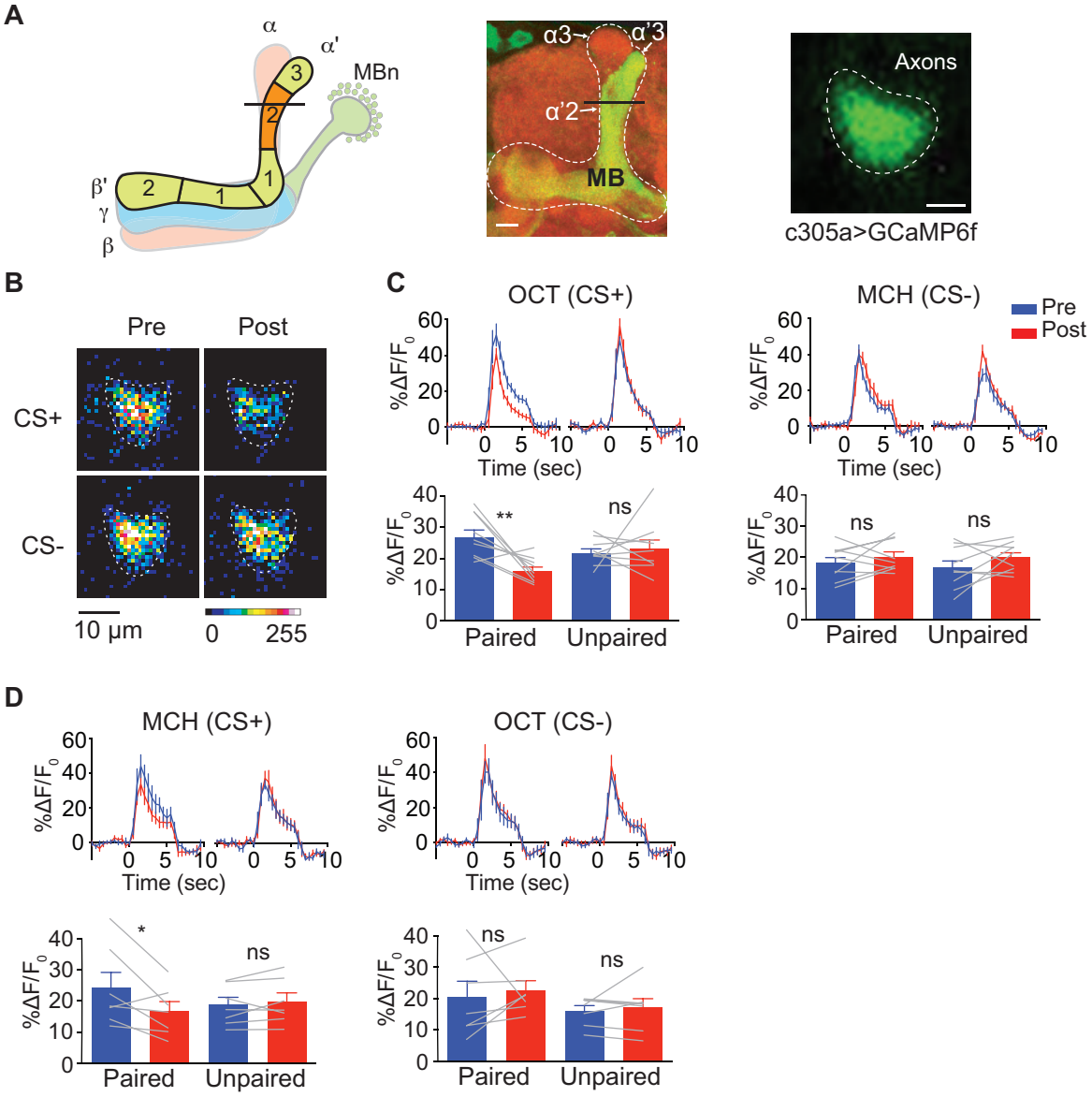


Figure 3

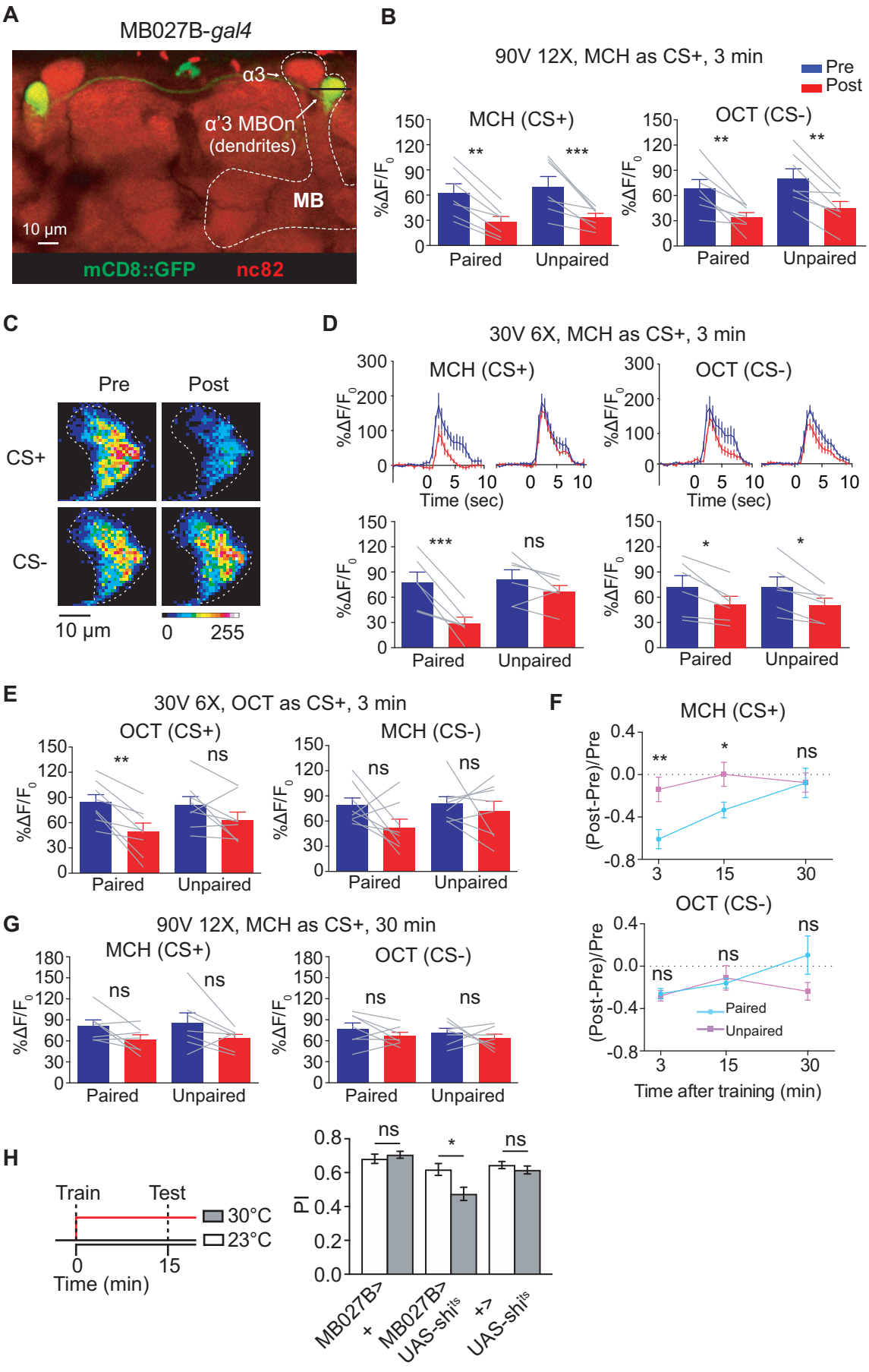


Figure 4

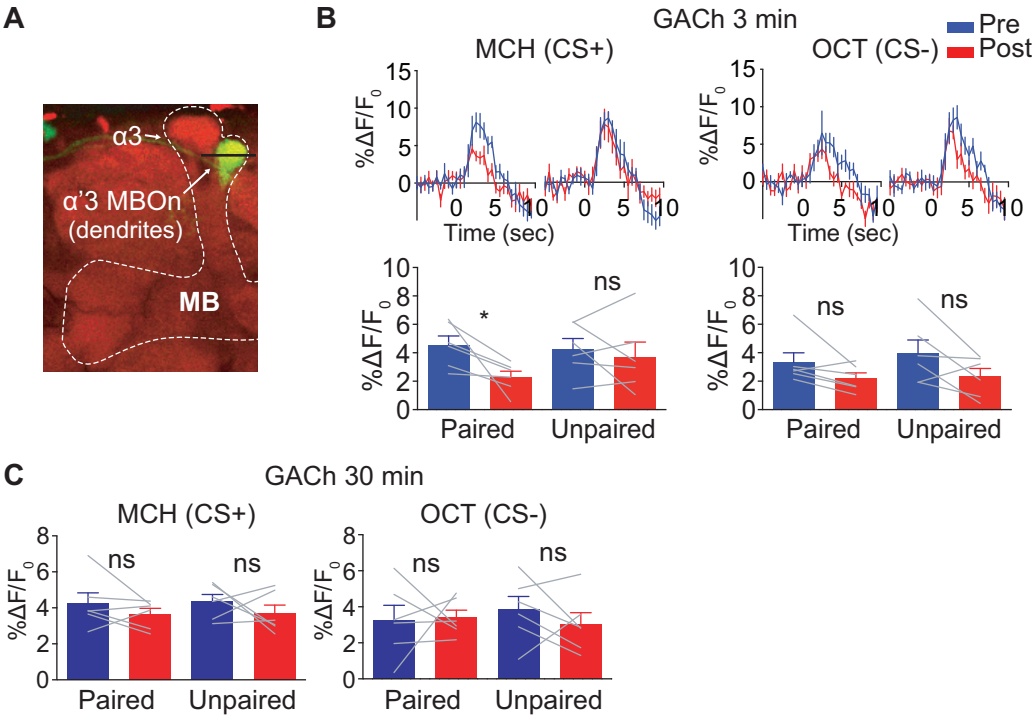


Figure 5

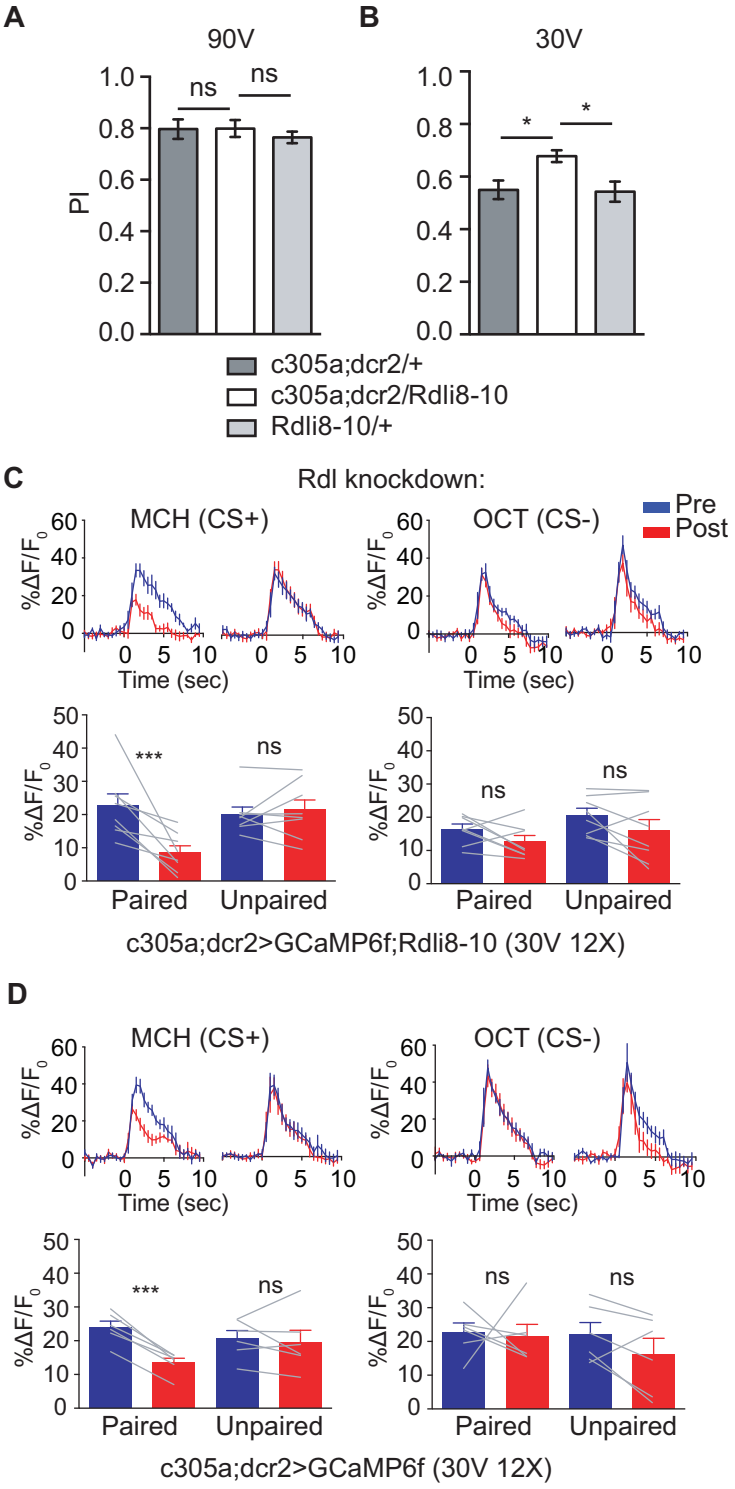


Figure 6