Behavioral/Systems/Cognitive

Imaging of an Early Memory Trace in the *Drosophila* Mushroom Body

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Extensive molecular, genetic, and anatomical analyses have suggested that olfactory memory is stored in the mushroom body (MB), a higher-order olfactory center in the insect brain. The MB comprises three subtypes of neurons with axons that extend into different lobes. A recent functional imaging study has revealed a long-term memory trace manifested as an increase in the Ca^{2+} activity in an axonal branch of a subtype of MB neurons. However, early memory traces in the MB remain elusive. We report here learning-induced changes in Ca^{2+} activities during early memory formation in a different subtype of MB neurons. We used three independent *in vivo* and *in vitro* preparations, and all of them showed that Ca^{2+} activities in the axonal branches of α'/β' neurons in response to a conditioned olfactory stimulus became larger compared with one that was not conditioned. The changes were dependent on proper G-protein signaling in the MB. The importance of these changes in the Ca^{2+} activity of α'/β' neurons during early memory formation was further tested behaviorally by disrupting G-protein signaling in these neurons or blocking their synaptic outputs during the learning and memory process. Our results suggest that increased Ca^{2+} activity in response to a conditioned olfactory stimulus may be a neural correlate of early memory in the MB.

Key words: learning and memory; *Drosophila*; imaging; calcium; olfactory; fluorescence microscopy

Introduction

Memory is generally considered to be encoded and stored as experience-dependent modifications of synaptic strength and structure (Kandel and Pittenger, 1999; Thompson, 2005). Where in the brain such modifications (i.e., the memory trace) take place is one of the key issues in memory research. In *Drosophila*, strong evidence suggests that olfactory memory is primarily stored in the mushroom body (MB) (Heisenberg, 2003; Gerber et al., 2004; Davis, 2005), a higher-order brain center that receives olfactory inputs from the antennal lobe (AL), which in turn receives inputs from the olfactory receptor neurons (Hildebrand and Shepherd, 1997; Hallem and Carlson, 2004). For example, chemical ablation of the MB (de Belle and Heisenberg, 1994) abolishes olfactory learning. Furthermore, targeted disruption of the G-protein signaling pathways (Connolly et al., 1996; Ferris et al., 2006) or cAMP cascades (Zars et al., 2000; McGuire et al., 2003) in the MB also disrupts olfactory learning. Finally, temporary blockage of the MB neuron output disrupts memory acquisition, consolidation, and retrieval (Dubnau et al., 2001; McGuire et al., 2001; Krashes et al., 2007).

Following their birth orders, the MB neurons can be divided into three subtypes: the γ, α'/β', and α/β neurons (Crittenden et al., 1998; Lee et al., 1999). Axons of the γ neurons form the horizontal γ lobe, whereas those of α'/β' and α/β neurons bifurcate and extend into vertical α' and α and horizontal β' and β lobes, respectively (see Fig. 1A). Emerging evidence suggests that different subtypes of MB neurons may be involved in different phases of the olfactory memory formation. The phases of olfactory memory include short-term memory (STM), middle-term memory (MTM), anesthesia-resistant memory (ARM), and long-term memory (LTM) (Tully et al., 1994). Behavioral studies have suggested that the α' and α lobes may be important for LTM (Pascual and Preat, 2001), whereas the γ lobe may play an important role in *rutabaga*-mediated STM (Zars et al., 2000). The outputs from α'/β' neurons are required during acquisition and consolidation, whereas the outputs from α/β neurons are required during memory retrieval (Krashes et al., 2007). Consistent with these findings, a functional imaging study has revealed an LTM trace in the α lobe of the α/β neurons (Yu et al., 2006). However, although an STM trace has been shown to exist in the AL (Yu et al., 2004), direct demonstration of an STM trace in the MB is still lacking.

In this study, we visualized learning-induced neural plasticity in the MB with functional Ca^{2+} imaging. We found that after a single training, Ca^{2+} responses in the α'/β' lobes were enhanced for trained odors compared with control odors. This enhancement lasted up to 1 h and could be blocked by disrupting G-protein signaling in the MB. Restricted disruption of G-protein signaling in the α'/β' neurons or blocking synaptic outputs from these neurons impaired the memory formation.

These results suggest that the enhanced Ca^{2+} responses in the α'/β' lobes represent an STM trace.

Materials and Methods

Transgenic flies and T-maze training. Flies carrying the *UAS-G-CaMP* transgene were crossed with the MB GAL4 line, *OK107*. Offspring from this cross (*UAS-G-CaMP×OK107*) have strong G-CaMP expression in the MB neurons. We also generated homozygous flies carrying both *UAS-G-CaMP* and an MB Gal4 driver, *238Y*. These flies (*238Y;UAS-G-CaMP*) were used interchangeably with *UAS-G-CaMP×OK107* flies. There was no difference in imaging and behavior results obtained from the two lines of flies. To express different variants of $G\alpha_s$ together with G-CaMP in the MB, *UAS-Gα_s** and *UAS-Gα_s^+* with a *2202U* genetic background were crossed with *238Y;UAS-G-CaMP*. *2202U× 238Y;UAS-G-CaMP* and *UAS-Gα_s^+×238Y;UAS-G-CaMP* were used as controls for *UAS-Gα_s*×238Y;UAS-G-CaMP*. *2202U* is the isogenized strain of wild-type w^{1118}.

Flies were trained in a standard T-maze as described previously (Tully and Quinn, 1985). Briefly, ~120 flies were sequentially exposed to a positive conditioned stimulus (CS$^+$) odor paired with footshock and then a negative conditioned stimulus (CS$^-$) odor without footshock. Flies were either tested immediately after training (3 min memory) or, with one fly caught alive for imaging, tested at 2 h after training. After the training, the flies were kept on standard *Drosophila* medium at room temperature. During testing, flies were exposed simultaneously to the CS$^+$ and CS$^-$ odors in a T-maze. A performance index (PI) was calculated from the distribution of flies in the two T-maze arms. A 50:50 distribution (no memory) gives rise to a PI score of 0, and a 0:100 distribution away from the CS$^+$ gives rise to a PI of 100. A 1000-fold dilution of octanol (OCT) and 2500-fold dilution of benzaldehyde (BA) in mineral oil were used. For control cases, flies were sequentially exposed to a CS$^+$ odor, electric shock, and a CS$^-$ odor, each for 1 min, with a 1 min interval in between.

Living fly imaging. The captured fly was prepared for live imaging as described previously with some modifications (Wang et al., 2001). Briefly, the fly was immobilized in a micropipette tip with its head exposed. A plastic coverslip with a small window was placed over the head and sealed onto the fly around the edges of the window with dental wax. After covering the head with adult fly saline [in mM: 115 NaCl, 5 KCl, 1.5 CaCl$_2$ · 2H$_2$O, 4 MgCl$_2$ · 6H$_2$O, 4 NaHCO$_3$, 1 NaH$_2$PO$_4$ · 1H$_2$O, 5 trehalose, 75 sucrose, and 5 N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid, pH 7.1, 356 mOsm], a tiny hole was cut through the cuticle to make the MB accessible for imaging.

To train a fly that was immobilized for imaging, the preparation described above was modified slightly. The micropipette tip that held the fly had a metal electrode inserted through which the electric shock could be delivered to the fly's abdomen. To ensure the shocking of the abdomen, the surface of the metal electrode was covered with electro-conductive gel. Training was done to the single fly following a procedure similar to the one used in the T-maze, except that 120 V square pulses were used instead of standard 60 V pulses. For unpaired control experiments, flies were sequentially exposed to BA, electric shock, and OCT, each for 1 min, with a 1 min interval in between. Odors were diluted in mineral oil at the same concentrations used in the T-maze training and were delivered to the fly's free-moving antennae by an air stream (400 ml/min) bubbled through mineral oil. Electric shock evoked very small responses in the MB, which could only be revealed after a spectral analysis of the averaged response time course.

A custom-built two-photon laser-scanning microscope was used. The objective was a 60× water-immersion lens (0.9 numerical aperture; Olympus, Center Valley, PA). The laser source was a Chameleon Ti:sapphire laser (Coherent, Santa Clara, CA) tuned to wavelength (λ) 910 nm. Images were acquired at ~5 Hz (0.2 s per image). Recordings were taken from single optical sections of the MB. At the end of each experiment, an XYZ scan (z step of 1 μm) was always performed to ascertain the identification of the MB substructures. The three-dimensional MB structure was reconstructed from such scans using Amira (TGS, San Diego, CA).

Isolated brain preparation. The brain was isolated from the cuticle with the antennal nerves (ANs) and the ventral nerve cord (VNC) attached and was pinned down on a thin Sylgard slab in a Petri dish. The electric stimulation to the AN and VNC were accomplished by two suction electrodes, both with a train of 10 pulses (single pulse duration, 1 ms) at 100 Hz using a Grass S88 stimulator (Grass Technologies, West Warwick, RI). For paired stimulation, 12 trains at an interval of 5 s were delivered simultaneously to the AN and VNC. For backward pairing, the stimulation of the VNC preceded that of the AN by 30 s. For unpaired stimulation, there was a 1 min interval between AN stimulation and VNC stimulation. Responses in the MB evoked by the stimulation of the AN was much bigger than those evoked by the *in vivo* odor stimulations. To ensure the response to AN and VNC stimulation was not saturated, voltages of stimuli were chosen to give a response that was below 30% of the maximum responses.

Image analysis. The ΔF images were generated as described previously (Wang et al., 2004). Briefly, frames acquired before the onset of odor stimulation were averaged, and the SEM of the background fluorescence fluctuation at each pixel was calculated. The averaged background image was subtracted from the average of a few frames at the peak of the odor response to obtain the ΔF image. A pixel-by-pixel threshold of two times the SEM of the background fluorescence fluctuation was applied to the ΔF image. Regions of interest were identified based on the basal fluorescence of G-CaMP showing characteristic shape and location of each region of interest. Each lobe was clearly identifiable at the tip region, and the x-y-z stack images were used to follow each lobe to the depths of recordings. The ratio of the peak response amplitude to the CS$^+$ odors versus CS$^-$ odors were log transformed (base 10) to normalize the distribution of the ratio, and statistical tests were performed on log transformed values. Unless noted otherwise, data were analyzed by ANOVA followed by Tukey–Kramer honestly significant difference *post hoc* tests, and the critical values for individual comparisons were adjusted to maintain an experiment-wise error rate of $\alpha = 0.05$.

Immunohistochemistry. Brains were dissected in PBS and fixed in 4% paraformaldehyde (in PBS) at room temperature for 10 min. After vacuuming twice in PBST (PBS with 0.2% Triton X-100) and 4% paraformaldehyde for 10 min, the brains were blocked with 10% normal goat serum (NGS) on ice for 2 h. The brains were then incubated overnight at 4°C with mouse 1D4 anti-Fasciclin II antibody (supernatant, 1:20 diluted in PBS with 0.25% Triton X-100, 1% NGS, and 0.02% NaN$_3$; DSHB, University of Iowa, Iowa City, IA). After washing four times in PBS, the brains were incubated overnight at 4°C with goat anti-mouse IgG conjugated with Alexa Fluor 647 (1:200 diluted in PBS with 0.25% Triton X-100, 1% NGS, and 0.02% NaN$_3$; Invitrogen, Eugene, OR). The brains were then washed and mounted in FocusClear (Pacgen, Vancouver, British Columbia, Canada). Confocal images of the brain were taken with an LSM 510 confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY).

Results

We used a genetically engineered calcium sensor, G-CaMP, to monitor neuronal activities (Wang et al., 2003, 2004). Expression of the *G-CaMP* transgene was targeted to the MB using the GAL4-UAS system (Brand and Perrimon, 1993). Two GAL4 lines showing strong expression in the MB, OK107 and 238Y (Connolly et al., 1996), were used interchangeably in this study. A fly was restrained in a holder fashioned from a micropipette tip, and a window was opened in the head for imaging with a two-photon laser-scanning microscope. The basal fluorescence of the G-CaMP allowed identification of the MB structure (Fig. S1, available at www.jneurosci.org as supplemental material). Recordings were made from optical sections in regions of the calyx (the dendritic region of MB neurons) and the vertical α'/α and horizontal $\beta'/\beta/\gamma$ lobes as illustrated in Figure 1*A*. Delivery of an odor evoked increases in G-CaMP fluorescence in the calyx and axonal branches (Fig. 1*B,C*). The majority of this rise in G-CaMP fluorescence is likely to be caused by an influx of extracellular Ca^{2+}, because depletion of the intracellular Ca^{2+} stores did not diminish G-CaMP signals evoked by stimulation of the olfactory pathway (Fig. S2, available at www.jneurosci.org as supplemental material). In the axonal branches, odor-evoked calcium re-

sponses were much higher in the α' and β' lobes than in α, β, and γ lobes (Figs. 1, 2).

We trained flies with an olfactory associative learning paradigm that has been most extensively characterized in flies (Tully and Quinn, 1985). In this paradigm, flies learn to avoid an odor that is associated with electric shock. We exposed flies first to an odor (CS$^+$), BA, which was paired with electric shock to the flies, followed by exposure to another odor (CS$^-$), OCT, without electric shock. In control groups, flies were exposed to the two odors and electric shock sequentially without pairing (Fig. 2A). After training, a fly was captured randomly without anesthetization and prepared for live imaging of Ca^{2+} responses in the MB to the CS$^+$ and CS$^-$ odors. The cohort flies were tested behaviorally after the completion of live imaging to demonstrate the memory retained that was evidenced by their ability to avoid the CS$^+$ odor (Fig. 2A). At 2 h after training, the flies still retained a significant level of memory. Constrained by the time needed to prepare a fly for live imaging, G-CaMP responses to both the CS$^+$ and CS$^-$ odors were recorded in captured flies at ~60 min after training. In trained flies, responses to BA relative to those to OCT were much enhanced in α'/β' lobes compared with control flies (Fig. 2C–E, Control and BA-trained). The time course of the G-CaMP response seemed not to have changed by training (Fig. 2B–D). No relative changes in responses to BA and OCT in the calyx and the α/β and γ lobes were observed (Fig. 2B–E). These findings were confirmed by switching the two odors in the T-maze training with OCT as CS$^+$ and BA as CS$^-$. After such training, activities in α'/β' lobes in response to OCT were enhanced compared with BA (Fig. 2C,D,F). No activity changes were observed in the calyx and the γ and α/β lobes (Fig. 2B–D,F). Our recordings covered large fractions of the calyx, β', β, and γ lobes, although in the vertical lobes (α' and α), recordings were restricted around the tip region of the α' lobe (Table S1, available at www.jneurosci.org as supplemental material), where most of the G-CaMP responses to olfactory pathway stimulation were found (data not shown). It is therefore unlikely that the lack of changes in G-CaMP response in the calyx and the γ and α/β lobes is attributable to inadequate sampling. We also used the Gal4 line *G0050* to express G-CaMP specifically in α'/β' neurons (Lin et al., 2007) and performed the same experiment to confirm the learning-induced increase in the odor-evoked activity in these lobes (Fig. S3, available at www.jneurosci.org as supplemental material). Results from these experiments confirmed that activities in α'/β' lobes in response to a CS$^+$ odor was enhanced compared with a CS$^-$ odor after the training (Fig. S3, available at www.jneurosci.org as supplemental material).

However, in the first preparation, activity in the MB immediately after learning could not be examined, and odor responses before and after training in a single fly could not be compared. To overcome these problems, we adopted a preparation that allowed training while a fly was being imaged (Yu et al., 2004). The fly was immobilized, and the head capsule opened up for Ca^{2+} imaging. Electric shock was delivered to the fly with a metal wire inserted under its abdomen. The fly was first exposed to BA paired with electric shock, followed by exposure to OCT without the shock. We examined responses to both odors in the vertical lobes before and after training. Odor-evoked responses were mostly in the α' lobe as were shown in the first preparation (Fig. 3). We found a trend of BA response to become larger and OCT response to become smaller after the training, but this trend was not statistically significant ($p > 0.05$). When we compared the ratio of BA response to OCT response, we found a statistically significant
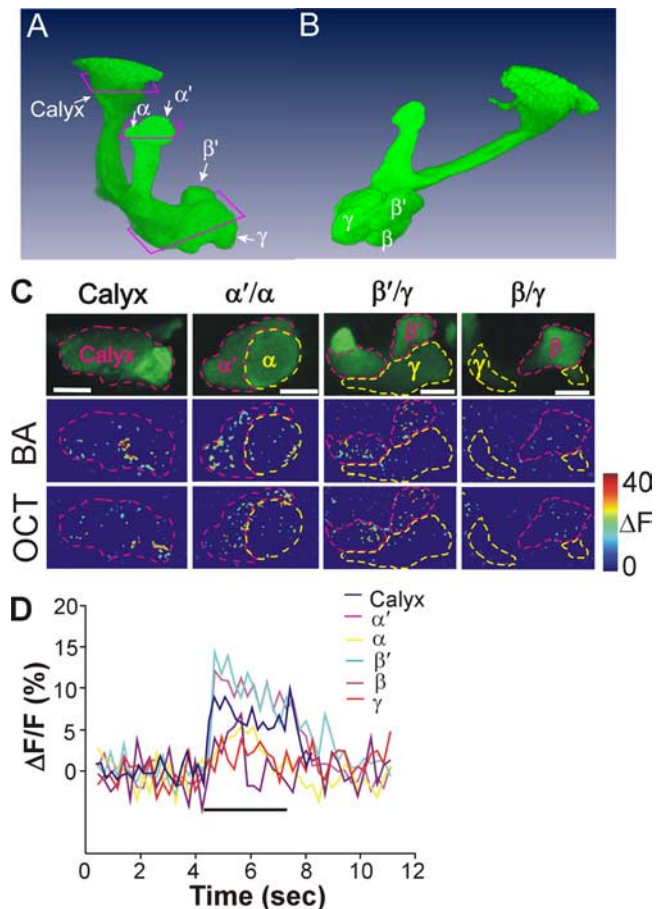

Figure 1. Odor-evoked G-CaMP responses in the MB. *A*, MB structure reconstructed from two-photon scanning stacks of a living fly brain expressing G-CaMP driven by OK107 (frontal view). The MB was identified by the basal G-CaMP fluorescence. Only the right half of the MB is shown. The arrowheads identify the axonal branches of subtypes of MB neurons. The rectangular outlines indicate places where recordings were taken to monitor activities in the calyx and different axonal branches. *B*, Sagittal view of the reconstructed MB structure. The β lobe lies beneath the γ and β' lobes. *C*, G-CaMP responses evoked by BA (second row) and OCT (third row) in the calyx and axonal branches of the MB. ΔF images of the G-CaMP response are shown. The top row shows basal G-CaMP fluorescence that identifies the structures from which recordings were made. *D*, Time course of the G-CaMP response to BA in the calyx and axonal branches of the MB outlined in *C*. The time course of G-CaMP response in only one of the sections of the γ lobe shown in *C* is plotted. The bar shows the duration of odor stimulation. Scale bar: vertical lobes, 10 μm; calyx and horizontal lobes, 20 μm.

increase in the α' lobe 60 min after training (Fig. 3B). There was also a slight increase at 30 min, although not statistically significant. No change in the response to BA and OCT was found in the control flies that were exposed sequentially to BA, electric shock, and OCT (repeated-measures ANOVA; $F_{(2,8)} = 0.38$; $p = 0.6933$). For both trained and control flies, no changes were observed in the α lobe. This is consistent with the finding obtained from flies trained in the T-maze.

In the second preparation, an immediate change in Ca^{2+} activities could have been masked by considerable brain movement that reduces the signal-to-noise ratio. To minimize the muscle movement, we developed another preparation that allowed us to investigate associative conditioning. We stripped bare the living fly preparation with only the brain left and the AN and VNC attached. We paired electric stimulations of the AN, which conducts olfactory input from the periphery, with that of the VNC, which may convey sensation of electric shock from the body to the brain, and imaged neuronal activities in the MB in response to
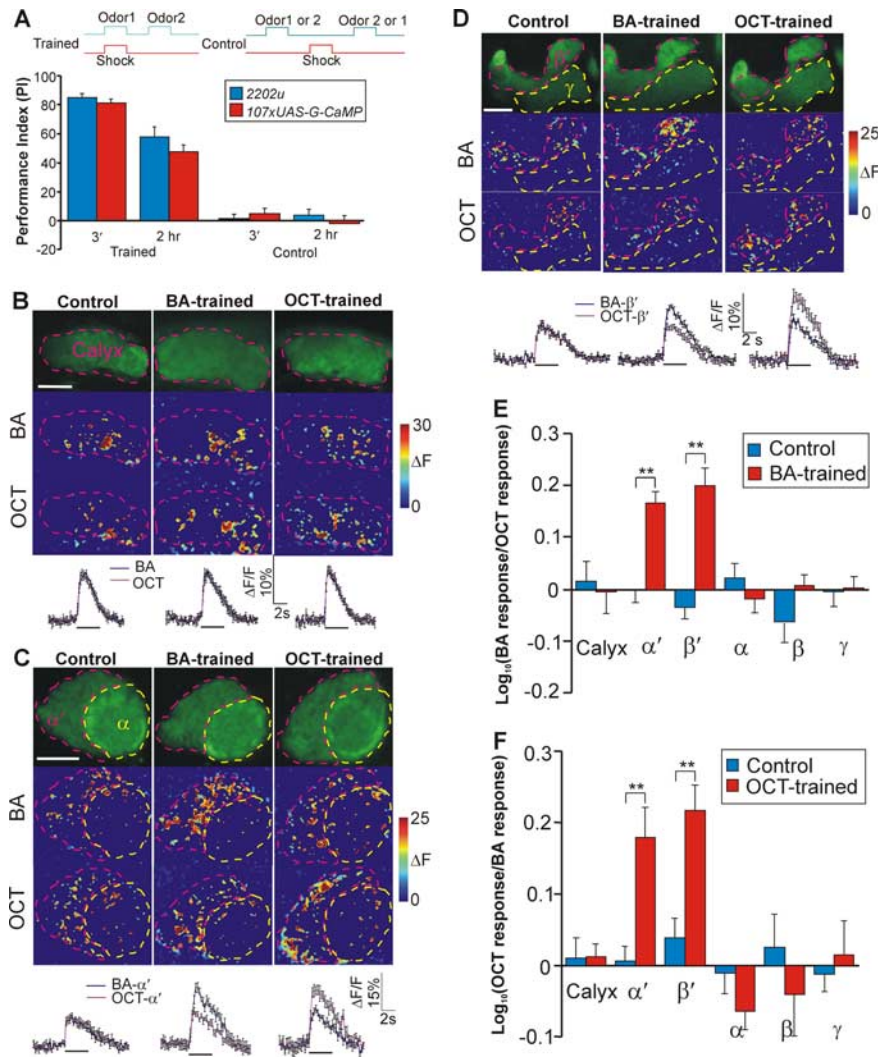
Figure 2. Learning-induced enhancement in G-CaMP response to CS $^+$ odors in the MB α' and β' lobes after olfactory training. **A**, Memory performance of flies immediately after training (3 min), or 2 h after. Flies were forced to choose between the CS $^+$ and CS $^-$. A score of 100 indicates all flies avoiding the CS $^+$. The training protocols are illustrated on top. Flies were either trained with BA (BA as CS $^+$ and OCT as CS $^-$) or OCT (OCT as CS $^+$ and BA as CS $^-$). In training, ~120 flies were first exposed to the CS $^+$ odor paired with electric shock for 1 min. After resting for 1 min, the flies were exposed to the CS $^-$ odor for 1 min. In control, about the same number of flies was sequentially exposed to CS $^+$, electric shock, and CS $^-$, each for 1 min, with a 1 min interval. After the training, the flies were either tested immediately for 3 min memory, or with a fly caught for live imaging, the rest were tested 2 h after the training. **B–D**, The G-CaMP response to BA (second row) and OCT (third row) in the calyx (**B**), α'/α (**C**), and β'/γ (**D**) lobes of the control (first column), BA-trained (second column), and OCT-trained (third column) flies. Recordings were made ~60 min after the training. In the lobes, responses to BA and OCT were mostly concentrated in the α'/β' lobes (**C, D**). The responses to the CS $^+$ odors were specifically enhanced relative to the responses to the CS $^-$ odors in the α' and β' lobes after training (**C, D**). No change was observed in the response to the CS $^+$ odors relative to CS $^-$ odors in flies trained with either BA or OCT in the calyx (**B**), α lobe (**C**), and γ lobe (**D**). The top rows show G-CaMP basal fluorescence that identified structures from where recordings were made. The bottom rows show averaged time courses of the G-CaMP responses ($\Delta F/F$) to BA and OCT from the corresponding group of flies in the MB regions outlined by the dashed lines. The number of flies used in each averaged time course ranged from 7 to 10. The bars indicate the duration of odor stimulation. **E, F**, Enhanced responses to the CS $^+$ relative to the CS $^-$ odors in the α'/β' lobes in BA-trained (**E**) and OCT-trained (**F**) flies. Recordings were made in the calyx, α', α, β', β, and γ lobes. The Log ratios of the CS $^+$ response to the CS $^-$ response were calculated using the peak amplitudes of the responses. In **E**, n = 7 and 9 for control and BA-trained flies, respectively. In **F**, n = 8 and 10 for control and OCT-trained flies, respectively. Asterisks indicate significance between trained and control flies (*$p < 0.05$; **$p < 0.01$). Error bars represent SEM. Scale bars: vertical lobes, 10 μm; calyx and horizontal lobes, 20 μm.

AN stimulation before and after pairing. Although the pathway that carries the electric shock information to the brain is not clear, we think that the stimulation of the VNC provides a good approximation. It evoked responses in the MB similar to those shown by others when the electric shock was delivered to the

abdomen (Yu et al., 2006). Stimulations of the AN or VNC both evoked activities in the MB calyx and lobes (Fig. 4A). Brain movement was drastically reduced in this preparation as shown by the smoother baseline in the time course plot (Fig. 4A). A paired stimulation of the AN and VNC that mimicked the time course of the olfactory training in the T-maze enhanced Ca^{2+} responses in α'/β' lobes to a subsequent AN stimulation at ~3 min after pairing (Fig. 4B,C). No such enhancements were detected in the calyx and the γ and α/β lobes (Fig. 4B). Backward pairing in which VNC stimulation preceded AN stimulation, or sequential stimulation of the AN and the VNC without any overlap, did not change responses to AN stimulation (Fig. 4D,E). This shows that the enhanced responses to AN stimulation is specific to the paired stimulation of the AN and VNC.

It has been shown that associative olfactory learning can be disrupted by expressing in the MB a mutant form of the stimulatory heterotrimeric G-protein α subunit ($G\alpha_s^*$) that constitutively activates the adenylyl cyclase and impairs the cAMP pathway (Connolly et al., 1996). The same study also showed that expression of the wild-type $G\alpha_s$ ($G\alpha_s^+$) does not affect learning. If a pairing of the AN and VNC stimulation truly mimicked olfactory learning, the pairing-induced enhancement of responses to AN stimulation in α'/β' lobes should be blocked by the expression of $G\alpha_s^*$ in the MB, but not with the expression of $G\alpha_s^+$ in the MB. We expressed both G-CaMP and $G\alpha_s^*$ or $G\alpha_s^+$ in the MB and compared responses to AN stimulation before and after the pairing in the MB. Although responses to AN stimulation was enhanced after the pairing in α'/β' lobes in control flies and in flies expressing $G\alpha_s^+$, such enhancement was absent in flies expressing $G\alpha_s^*$ (Fig. 5). So, indeed, the disruption of the G-protein signaling pathway in the MB abolished the pairing-induced enhancement of responses to AN stimulation in α'/β' lobes, further supporting the idea that the pairing-induced enhancement is a result of an associative process.

The enhanced G-CaMP response to trained odors compared with control odors in α'/β' lobes after training indicates that α'/β' neurons may be involved in the learning and memory process. This is consistent with a recent finding that the output from α'/β' neurons are required for acquisition and consolidation of 3 h memory (Krashes et al., 2007). To further demonstrate the participation of α'/β' neurons in early forms of memory, we disrupted the G-protein signaling only in α'/β' neurons by targeting the constitutively active $G\alpha_s^*$ to these neurons with the GAL4 line *G0050* (Lin et al., 2007) (Fig.
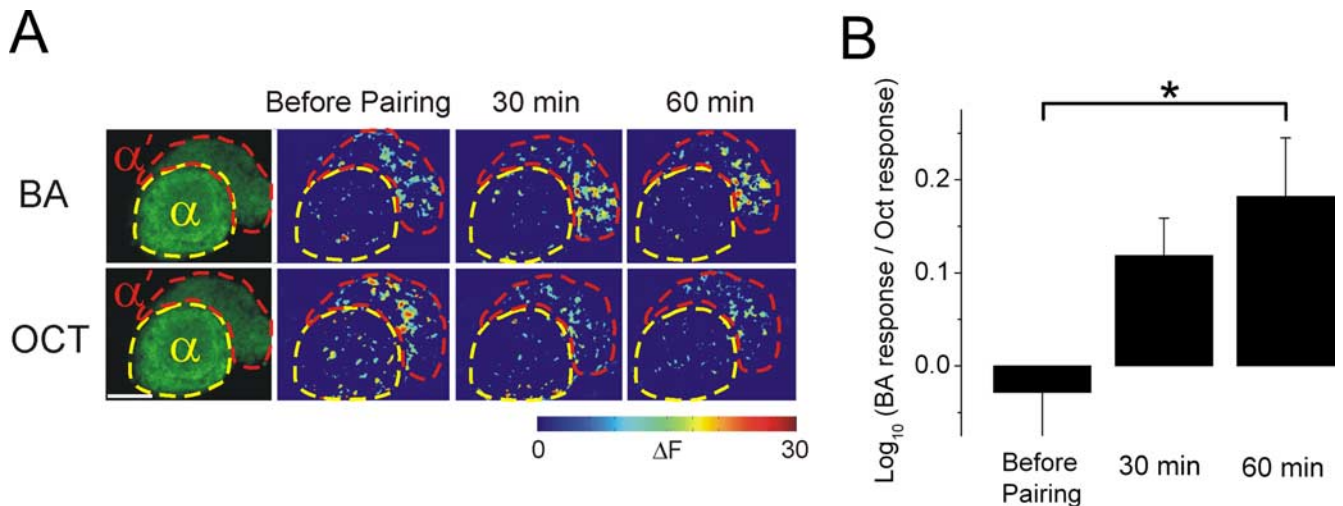
A

B

Figure 3. Pairing BA with electric shock changes the ratio of G-CaMP response to BA and OCT in the α' lobe of the MB. **A**, First column, The basal fluorescence of *G-CaMP* expressed with *OK107-GAL4* in the α (surrounded by yellow dotted lines) and α' (surrounded by red dotted lines) lobes of the MB (scale bar, 10 μm). Note that the lateral is to the right in these images. Second to fourth columns, Changes in *G-CaMP* fluorescence (ΔF) in response to 3 s presentations of BA (top row) and OCT (bottom row) at different time points before and after the pairing of BA with electrical shock (second column, before pairing; third column, 30 min after the pairing; fourth column, 60 min after the pairing). Most of the changes in G-CaMP fluorescence were seen in the α' lobe. In this example, after the pairing, responses to BA became slightly bigger, whereas responses to OCT became slightly smaller. **B**, Log_{10} of the response to BA relative to the response to OCT changed significantly after the pairing (one-way ANOVA; $F_{(2,15)} = 3.88$; $p = 0.0439$). Log_{10} of the response to BA relative to the response to OCT was calculated in each fly at various time points before and after the pairing of BA with electrical shock, and a mean value for six flies is plotted for each time point. Comparison of individual means showed that the log_{10} of the response ratio was significantly greater at 60 min after the pairing compared with the presentation before the pairing (Tukey–Kramer adjusted, *$p = 0.0328$). The peak amplitudes of the responses were used for the calculation of the response ratio. Error bars represent SEM.

$6A$). Memory was tested immediately after a single-cycle training using the standard T-maze training paradigm. As shown in Figure $6B$, disruption of the G-protein signaling in α'/β' neurons partially impaired memory immediately after the training. We also blocked outputs from the α'/β' neurons with a temperature-sensitive dynamin, Shi^{ts}, which can disrupt synaptic transmission in a dominant-negative way at restrictive temperatures (Kitamoto, 2001). Similar to disrupting the G-protein signaling, blockade of output from α'/β' neurons with Shi^{ts} by raising the temperature to 32°C partially impaired immediate memory (Fig. $6C$). We further examined the involvement of α'/β' neurons in 1 h memory which, like 3 h memory, comprises MTM and ARM. Consistent with the finding that the output from α'/β' neurons is required for consolidation of 3 h memory, blocking the output from α'/β' neurons after training impaired 1 h memory (Fig. $6D$). Task-relevant sensory-motor responses were not altered by either the expression of $G\alpha_s^*$ in the α'/β' neurons or disrupting the output from α'/β' neurons with Shi^{ts} at the restrictive temperature (Table S2, available at www.jneurosci.org as supplemental material).

Discussion

We used functional Ca^{2+} imaging in three different preparations to search for memory traces in the MB after a single-cycle training that produces STM, MTM, and ARM. Flies were trained in groups in the standard T-maze or individually while held immobilized. These two different settings in training both led to enhanced Ca^{2+} activity in response to trained odors in the axonal branches of the α'/β' neurons. The enhancement was specific to the odor that was paired with electric shock and was detected at ~1 hour after conditioning. The semi-*in vivo* preparation that mimicked the single-cycle olfactory training with paired stimulation of the AN and VNC led to a similar observation. In addition, an immediate enhancement in Ca^{2+} response in α'/β' lobes after the paired event was observed in this preparation. Targeted

disruption of the G-protein signaling in the MB blocked the enhancement of Ca^{2+} response after paired stimulation of AN and VNC. These results indicate that the enhanced Ca^{2+} response in α'/β' lobes after conditioning may represent a memory trace associated with early forms of memory. This was further confirmed in a behavioral experiment in which the 3 min and 1 h memory was impaired by disrupting the G-protein signaling in the α'/β' neurons or blocking synaptic output from these neurons.

Our finding does not preclude the possible existence of memory traces in other output branches of the MB neurons. There are a number of possibilities why the enhanced Ca^{2+} response to trained odors was only observed in axonal branches of the α'/β' neurons. Memory traces in the α/β and γ neurons may appear in forms other than alteration of Ca^{2+} response. It is known that a Ca^{2+}-independent PKC signaling can potentiate synaptic transmission presynaptically (Stevens and Sullivan, 1998). Another possibility is that for α/β and γ neurons, memory traces may occur postsynaptically in downstream neurons. Postsynaptic mechanisms play a key role in long-term potentiation and long-term depression (Malinow and Malenka, 2002). A third possibility is that the low G-CaMP responses in α/β and γ lobes are making it difficult to detect changes in these lobes (Figs. 1, 2). Activities in MB neurons are sparse (Perez-Orive et al., 2002; Wang et al., 2004), and the γ neurons tend to show a lower probability of response (Turner et al., 2008). Changes in a small amount of activity may be hard to detect with our imaging approach (Reiff et al., 2005). Finally, learning may lead to changes in the fine temporal pattern of neuronal activities in the MB that cannot be detected by Ca^{2+} imaging.

Olfactory learning and memory in *Drosophila* seem to involve multiple brain structures in parallel and sequential pathways. Blocking the output from the MB impairs retrieval of memory at multiple stages that encompass STM, MTM, ARM, and LTM
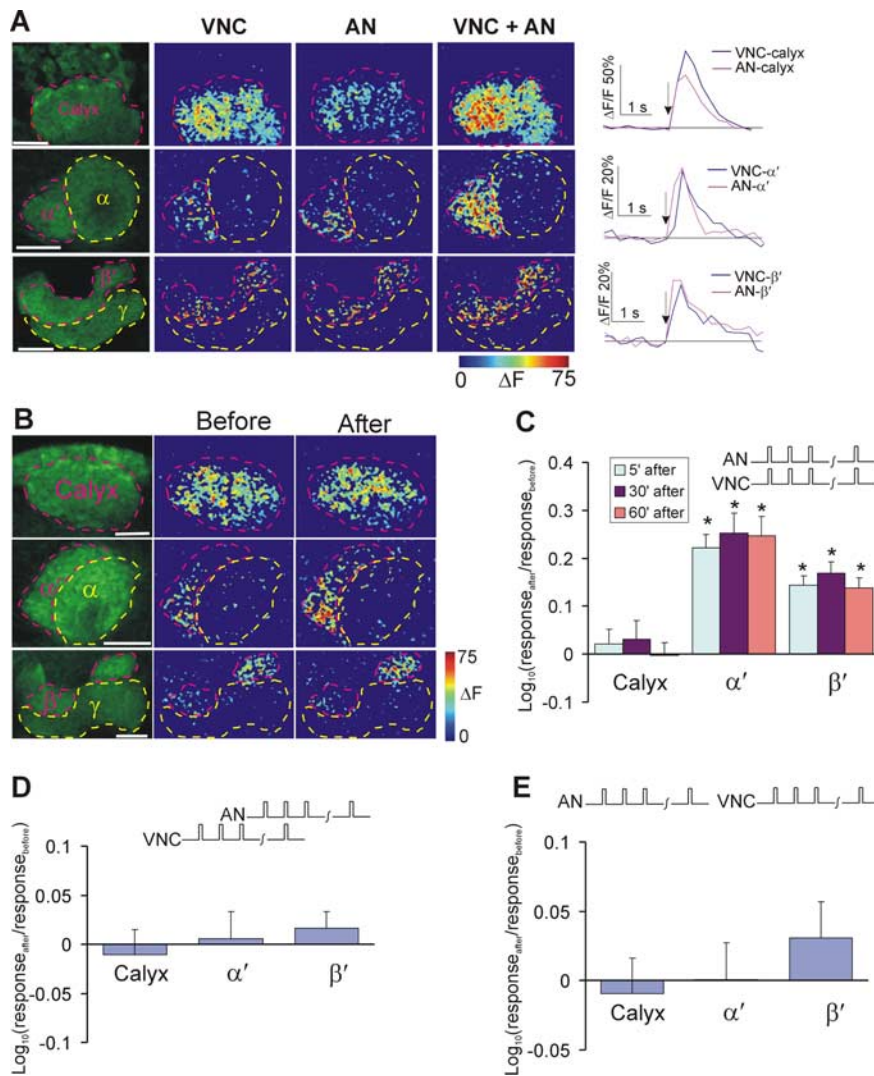
Figure 4. Enhancement in response to AN stimulation in the α'/β' lobes after paired electric stimulation of the AN and VNC. ***A***, Responses to stimulation of the AN and VNC in the MB. The basal G-CaMP fluorescence images (first column) reveals sections of the calyx, the vertical lobes (α' and α), and the horizontal lobes (β' and γ) where recordings were made. Second column, Response to VNC stimulation; third column, response to AN stimulation; fourth column, response to stimulation of both the AN and VNC; fifth column, time course of responses to AN and VNC stimulation in MB regions outlined by dashed lines. Arrows indicate when electric stimulations were delivered. Stimulations of the AN and VNC both evoked responses in the MB calyx and the vertical and horizontal lobes. Similar to responses to odor stimulation in the *in vivo* fly preparations, responses to AN and VNC stimulation in the isolated brain were mostly concentrated in the α'/β' lobes. ***B***, Responses to stimulation of the AN before and 5 min after paired electrical stimulation of the AN and VNC. Responses to AN stimulation before and 5 min after paired stimulation of the AN and VNC from the sections in the left column are displayed as ΔF images (two right columns). For stimulation of the AN and VNC, a train of 10 pulses (single-pulse duration, 1 ms) at 100 Hz was used. For paired stimulation, 12 trains at an interval of 5 s were delivered simultaneously to the AN and VNC. ***C***, Summary of the changes in responses to AN stimulation (expressed as $\log_{10}$ of response after pairing relative to that before) at different time points after paired stimulation of the AN and VNC. Significant enhancement in the response to AN stimulation occurred in the α'/β' lobes (*t* test, *$p < 0.001$; $n = 10$). The peak amplitudes of responses were used for the calculation of the response ratio. ***D, E***, Summary of the changes in responses to AN stimulation after a backward pairing of stimuli (***D***) to the AN and VNC (stimulation of the VNC preceded that of the AN by 30 s) or sequential stimulation of the AN and VNC (1 min interval) without overlap (***E***). No enhancement in responses to AN stimulation was observed ($n = 9$). Error bars represent SEM. Scale bars: (in ***A, B***) vertical lobes, 10 μm; calyx and horizontal lobes, 20 μm.

is required for acquisition and consolidation (Krashes et al., 2007). Several places outside the MB are also involved in olfactory learning and memory. Output from a pair of neurons called dorsal paired medial (DPM) neurons that innervate the entire MB lobes are required for 3 h memory in a time window of 30–150 min after conditioning (Keene et al., 2004, 2006; Yu et al., 2005). One of the NMDA receptor subunits, NR1, which is preferentially expressed in a small number of neurons that innervate the MB dendritic region, affects learning and LTM consolidation (Xia et al., 2005). Several genes expressed in a small number of neurons outside the MB were identified in a large-scale mutagenesis to affect 1 d memory (Dubnau et al., 2003). Dopaminergic and octopaminergic neurons, which both innervate the MB lobes, are believed to carry the aversive and appetitive unconditioned stimulus reinforcement, respectively (Schwaerzel et al., 2003).

Correspondingly, learning and memory-associated cellular changes, collectively called memory traces, have been found in some of these structures at different stages of memory processing. In DPM neurons, odor-evoked Ca^{2+} response in the branch that innervates the MB vertical lobes (α and α') is enhanced 30 min after conditioning (Yu et al., 2005). In the MB, enhanced odor-evoked Ca^{2+} response is observed in the α lobe 24 h after spaced training (multiple training sessions with a rest between sessions), which produces LTM (Yu et al., 2006). In addition, a memory trace appearing as recruitment of new projection neurons (PNs) in the AL occurs immediately after conditioning but lasts only a few minutes, disappearing at 7 min after conditioning (Yu et al., 2004).

How these memory traces interact with each other is not yet clear. The memory trace in the AL PNs and that we observed in the MB α'/β' neurons may appear in parallel after conditioning so STM may be formed in two independent places. The following observations support this possibility. One is that the memory trace in PNs is observed in the presynaptic specializations of PNs localized in the AL glomeruli (Yu et al., 2004). Second, we observed no change in the amount of odor-evoked

(Dubnau et al., 2001; McGuire et al., 2001; Isabel et al., 2004). This requirement of the MB output has been further dissected and assigned to different subtypes of MB neurons for different stages of memory processing. The initial study with the Shi[ts] has indicated that retrieval of 3 min and 3 h memory may be exclusively through α/β neurons (McGuire et al., 2001). The most recent study on 3 h memory confirmed the role for α/β neurons in retrieval and further revealed that output of the α'/β' neurons

Ca^{2+} response after olfactory training in the calyx, where synaptic connections are made between MB neurons and PNs. The spatial distribution of activity in the calyx was also not changed after olfactory training (data not shown). Therefore, the recruited new synaptic activities in the AL may not be transmitted to the MB to drive activity changes there at time points we studied.

The involvement of DPM neurons in memory consolidation relies on their projections to the axonal branches of the MB α'/β'

neurons (Keene et al., 2006). This suggests that memory consolidation may be performed by local interactions between DPM neurons and the MB α'/β' neurons, and the memory may reside in the α'/β' neurons during this process (Keene et al., 2006). This idea is also supported by the finding that the α'/β' neurons are required for memory acquisition and consolidation (Krashes et al., 2007). Our observation of a memory trace in the axonal branches of the MB α'/β' neurons also provides a strong support to the idea that the memory initially resides in the α'/β' lobes. From this perspective, it is interesting that the memory trace in the DPM neurons is only observed in branches that innervate the MB vertical lobes (Yu et al., 2005). It is not known whether this memory trace is restricted further to branches that innervate the α' lobe or is expressed in all branches that innervate vertical lobes. The memory trace in α'/β' neurons we observed here seems to appear before the memory trace in the DPM neurons, which was detected ~30 min after conditioning. Currently, we do not know whether there is any causal link between the two memory traces or whether they may exist independently. It should be interesting to see how the two memory traces are affected by manipulating activities of the DPM neurons and α'/β' neurons.

The LTM trace in the α lobe of the α/β neurons start to appear at 9 h after spaced training (Yu et al., 2006). It will be interesting to investigate whether the early memory trace we observed in the α'/β' neurons also exists after spaced training and how the STM trace is converted into the LTM trace. The LTM trace is blocked by mutation of the amnesiac gene (Yu et al., 2006), which is expressed in the DPM neurons (Waddell et al., 2000). Given the facts that the amnesiac mutation also blocks the intermediate memory trace in DPM neurons and that DPM projections to the axonal branches of the α'/β' neurons are sufficient for its role in memory consolidation, there is a very good possibility that the amnesiac mutation will disrupt the memory trace in the α'/β' neurons and this disruption results in the elimination of the LTM trace in the α/β neurons. Future studies addressing these issues should help us better understand how memory is formed and maintained in the brain.

Figure 5. Enhanced response to AN stimulation in α'/β' lobes after a paired stimulation of the AN and VNC is abolished by expression of the constitutively active $G\alpha_s$ ($G\alpha_s^*$). G-CaMP, together with $G\alpha_s^*$ or the wild-type $G\alpha_s^+$ was expressed in the MB under the control of the MB Gal4 driver 238Y. **A**, Response to AN stimulation in a fly expressing $G\alpha_s^*$ (*238Y;UAS-G-CaMP* × $G\alpha_s^*$) in the MB. First column, basal G-CaMP fluorescence showing locations in the MB where recordings were made. Second and third columns, G-CaMP response (ΔF) to AN stimulation recorded from regions outlined in the first column before and after pairing. **B**, Response to AN stimulation in a fly expressing $G\alpha_s^+$ (*238Y;UAS-G-CaM* × $G\alpha_s^+$) in the MB. Recordings were made from locations in the calyx (top row), α'/α (middle row), and β'/γ lobes (bottom row) outlined in the first column. Responses to AN stimulation were enhanced in α' and β' lobes after the pairing (third column) compared with before the pairing (second column). **C**, Summary of the changes in responses to AN stimulation after paired stimulation of the AN and VNC in control flies (*238Y;UAS-G-CaMP* × *2202u* and *238Y;UAS-G-CaMP* × *UAS-Gα_s^+*) and flies expressing $G\alpha_s^*$ (*238Y;UAS-G-CaMP* × *UAS-Gα_s^**). The enhancement observed in the α'/β' lobes in control flies (*t* test, $p < 0.05$) is absent in flies expressing $G\alpha_s^*$ ($n = 5$ for each group). The peak amplitudes of the responses were used to calculate the Log ratio of the responses. Error bars represent SEM. Scale bars: (in **A**, **B**) vertical lobes, 10 μm; calyx and horizontal lobes, 20 μm.

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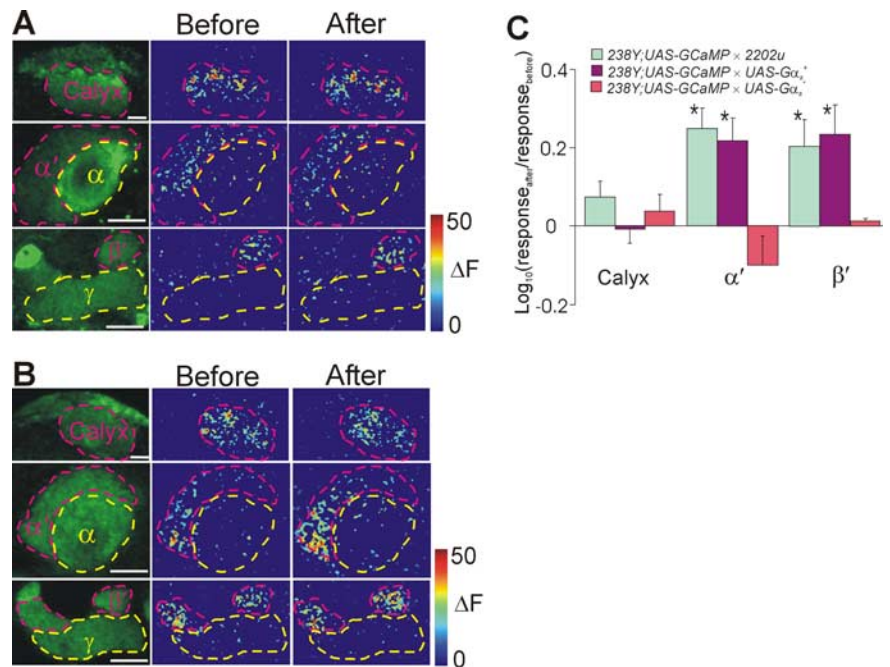abolished by chemical ablation of mushroom bodies. Science 263:692–695.

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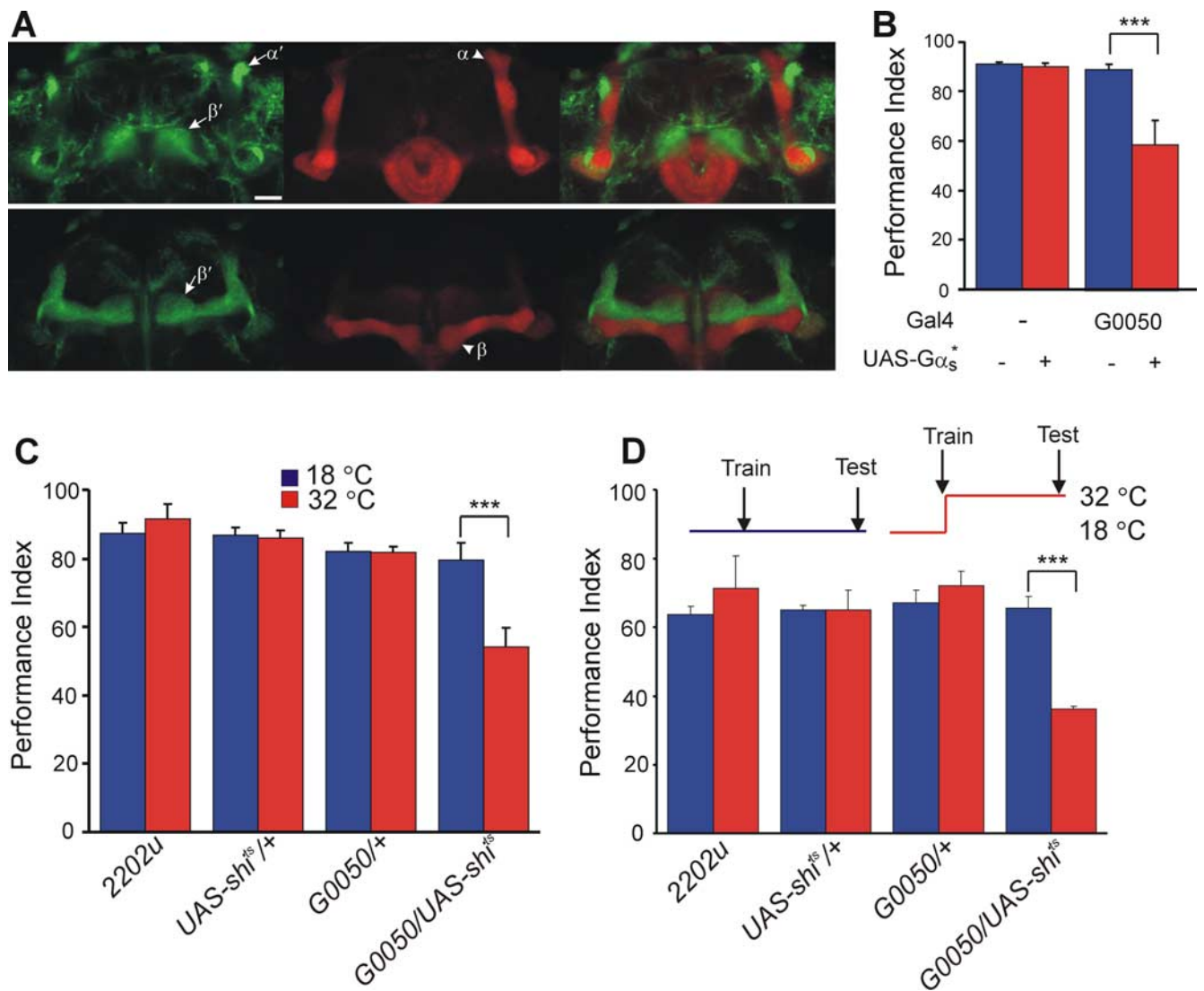Kitamoto T (2001) Conditional modification of behavior in *Drosophila* by

Figure 6. Disruption of the G-protein signaling in α'/β' neurons or blockade of their synaptic output impairs early memory. **A**, Green fluorescent protein (GFP) expression in the α'/β' lobes driven by the GAL4 line G0050. First column, Two optical sections at the MB lobe region showing GFP expression. The arrows point to the α'/β' lobes. Second column, The same optical sections in the first column showing FASII counterstain that labels the α/β and γ lobes in the MB (Crittenden et al., 1998). The arrowheads identify the α/β lobes. Third column, Merged images of the GFP fluorescence and FASII staining. In the MB, G0050 drives GFP expression only in the α'/β' lobes. GFP expression is also seen outside the MB, but no expression is observed in the AL and central complex. Scale bar, 20 μm. **B**, Impairment of immediate memory by disrupting the G-protein signaling in MB neurons. Expression of constitutively active Gα_s* only in α'/β' neurons impaired immediate (3 min) memory (***$p < 0.0001$; $n = 5$). **C**, Impairment of immediate (3 min) memory by disrupting synaptic output from α'/β' neurons. At a restrictive temperature, expression of the temperature-sensitive Shi^{ts} only in α'/β' neurons impaired immediate memory (***$p < 0.0001$; $n = 5$). **D**, Impairment of 1 h memory by disruption of synaptic output from α'/β' neurons. Flies were shifted to 32°C after training (red bars). In control groups, flies were kept at the permissive 18°C temperature (blue bars). Blocking synaptic output only in α'/β' neurons after training impaired 1 h memory (***$p < 0.0001$; $n = 5$). Error bars represent SEM.

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