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Gilgamesh Is Required for *rutabaga*-Independent Olfactory Learning in *Drosophila*

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SUMMARY

Cyclic AMP signaling in *Drosophila* mushroom body neurons, anchored by the adenylyl cyclase encoded by the *rutabaga* gene, is indispensable for olfactory memory formation. From a screen for new memory mutants, we identified alleles of the *gilgamesh* (*gish*) gene, which encodes a casein kinase Iγ homolog that is preferentially expressed in the mushroom body neurons. The *gish*-encoded kinase participates in the physiology of these neurons underlying memory formation since the mutant memory deficit was rescued with expression of a *gish* cDNA in these neurons only during adulthood. A cellular memory trace, detected as increased calcium influx into the α′/β′ neuron processes in response to the odor used for conditioning, was disrupted in *gish* mutants. Epistasis experiments indicated a lack of genetic interactions between *gish* and *rutabaga*. Therefore, *gish* participates in a *rutabaga*-independent pathway for memory formation and accounts for some of the residual learning that occurs in *rutabaga* mutants.

INTRODUCTION

The formation of new memories occurs, in part, through the activation of molecular signaling pathways within neurons that comprise the neural circuitry necessary for learning, storing memories, and expressing those memories when appropriate retrieval cues appear. A long-standing problem in the field of memory research is to define each of these pathways and how the activation of these pathways is translated into memory at the cellular and behavioral levels. Past research has implicated many signaling systems that participate in the cellular mechanisms underlying learning. For instance, calcium-mediated signaling is critical, in part through calmodulin dependent kinases, for the formation of cellular models for memory like hippocampal long-term potentiation (LTP) as well as for behavioral memory (Lisman et al., 2002). Signaling through mitogen activated protein kinases (MAPK) has also been shown to be necessary in hippocampal neurons for LTP and behavioral memories (Sweatt, 2004). Another major mechanism for forming memory is through cAMP signaling, a conclusion made across species early in the study of the cellular mechanisms of learning (Brunelli et al., 1976; Byers et al., 1981; Wong et al., 1999). In *Drosophila*, for instance, the molecular and biochemical characterization of two olfactory learning defective mutants, *dunce* and *rutabaga* (*rut*), demonstrated that the cAMP signaling pathway plays an essential role in memory formation. The *dunce* and *rut* genes encode a cAMP phosphodiesterase (Chen et al., 1986; Qiu and Davis, 1993) and a calcium:calmodulin-dependent adenylyl cyclase respectively (Levin et al., 1992). The normal products of these genes are preferentially expressed and required in the adult mushroom body neurons for normal learning (Nighorn et al., 1991; Han et al., 1992; Zars et al., 2000; McGuire et al., 2003; Mao et al., 2004). The delineation of the neurons that require these gene products, and the developmental period requiring their activity (adulthood), allows the very specific conclusion that cAMP signaling in adult mushroom body neurons underlies olfactory learning.

Although the cAMP pathway is essential for memory formation, the lack of either the *rut*-encoded cyclase or downstream protein kinase A (PKA) activity does not abolish initial learning after olfactory conditioning in *Drosophila* (Han et al., 1992; Skoulakis et al., 1993). Flies carrying strong hypomorphs or null alleles perform about half as well as control flies. This observation predicts the existence of additional molecules and independent signaling pathways that contribute to short-term memory formation. Consistent with this prediction, several other *Drosophila* short-term memory mutants have been discovered through genetic screens, including *Volado*, which encodes an α-integrin (Grotewiel et al., 1998); *fasII*, which encodes a cell adhesion receptor (Cheng et al., 2001); and *Leonardo*, which codes for a 14-3-3 protein (Skoulakis and Davis, 1996). Dopamine and NMDA receptors have also been shown to be involved in olfactory learning through candidate gene approaches (Tempel et al., 1984; Schwaerzel et al., 2003; Xia et al., 2005; Kim et al., 2007). However, it remains unknown whether these learning and memory genes define functions independent of cAMP signaling for memory formation. The delineation of all signaling pathways that are engaged in specific neurons during memory formation and how they interact with one another to encode memories is a general issue that needs resolution for a deep understanding of memory.

Genes that are required for memory formation may also have important roles in development. Homozygous mutants of these genes may not survive for the screening of adult behavioral deficits. Prior screens for learning mutants were all designed to detect recessive alleles and so genes with both essential

developmental and physiological (adult) roles would have been missed in prior screens. Although many of the early learning mutants were identified in recessive screens, subsequent characterization showed them to have dominant or semidominant effects on behavior (Davis, 1996; Grotewiel et al., 1998). This prompted us to conduct a dominant screen for genes that are important for memory formation. Here, we report the isolation of a new *Drosophila* memory gene, *gilgamesh* (*gish*). The *gish* gene encodes a casein kinase Iγ (CKIγ) homolog in flies (Hummel et al., 2002). The lack of genetic interactions between *gish* and *rutabaga* indicates that *gish* function in olfactory learning is independent of the cAMP signaling pathway headed by the *rut*-encoded adenyly cyclase.

RESULTS

Gish Is Required for Olfactory Learning

We have previously constructed and screened ~6000 enhancer detector lines for preferential expression of the *lacZ* reporter in the mushroom bodies (Han et al., 1996), because of the importance of these neural structures for olfactory learning in insects. Twenty of the lines from this expression screen were homozygous lethal or sterile. We tested memory retention in each of these lines as heterozygotes at multiple times after olfactory classical conditioning. One line, MB896, exhibited a significant impairment relative to the *rosy* (*ry*) control flies at 3 min, 30 min, and 3 hr after training (Figure 1A). In addition, homozygous MB896 flies, although sterile, exhibited a more severe impairment over heterozygotes in 3 min and 3 hr memory after olfactory conditioning (Figure 1B). The memory deficits in MB896 heterozygous and homozygous flies are unlikely due to defects in sensorimotor processes since no abnormality in shock or odor avoidance was observed in these flies (Figure 1C). Inverse PCR experiments defined the genetic location of the MB896 enhancer detector element to a site within the *gilgamesh* (*gish*) gene, which encodes a casein kinase Iγ (CKIγ) homolog in flies. Thus, the $gish^{MB896}$ line defined *gish* as a new, semidominant learning mutant with reporter expression preferentially in the mushroom bodies.

Gish Is Independent of *rutabaga*

Gish belongs to the CKI family of serine/threonine protein kinases. The major consensus phosphorylation sequence in substrate proteins for CKI family members is S/T(P)-X_{1-2}-S/T (Flotow et al., 1990; Meggio et al., 1992). The second S/T site becomes a CKI phosphorylation site only after the first S/T site is phosphorylated by a priming kinase. The substrate requirement for a priming phosphorylation suggests that CKI functions downstream of yet another protein kinase. Since the cAMP signaling cascade has been shown to be essential for normal learning and memory in *Drosophila*, we hypothesized that cAMP-dependent protein kinase (PKA) may function as the priming kinase for Gish function in learning. Since complete loss-of-function mutants of PKA are lethal (Skoulakis et al., 1993), we investigated the epistatic relationship between *gish* and the cAMP signaling pathway using alleles in the *rut*-encoded adenylyl cyclase gene, which functions upstream of PKA. First, we combined the rut^{2080} allele with $gish^{MB896}$ and a more extreme *gish* allele, $gish^{KG03891}$ (see below), respectively in the

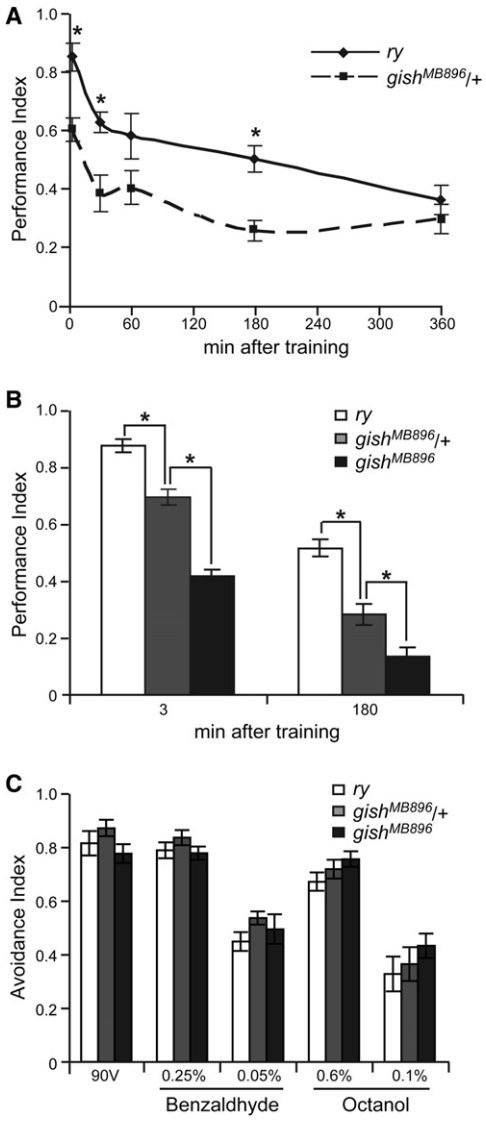

Figure 1. Memory Deficits in $gish^{MB896}$

(A) Performance decay after olfactory associative conditioning for $gish^{MB896}$ heterozygotes and the control strain (*ry*). n = 6 for each group. The performance of $gish^{MB896}$ heterozygotes was significantly different from the control at 3, 30, and 180 min after training (Bonferroni-Dunn; p = 0.0005, 0.0006, 0.0006, respectively), but not at 60 min (p = 0.0111) or 360 min (p = 0.3404).

(B) Semidominant effect of the $gish^{MB896}$ insertion. The $gish^{MB896}$ heterozygotes, homozygotes, and control flies were tested for memory retention 3 and 180 min after olfactory conditioning. n = 8 for each group. The homozygous $gish^{MB896}$ mutants performed significantly different than the heterozygous $gish^{MB896}$ flies at both time points tested (Bonferroni-Dunn; $p < 0.0001$ and p = 0.0010 for 3 min and 180 min respectively).

(C) Shock and odor avoidance of $gish^{MB896}$ heterozygotes, homozygotes and the *ry* controls. Flies were challenged with 90 V shock versus no shock, or an odor at one of several different concentrations versus a stream of fresh air and required to make a binary choice. n = 10 for each group. No significant difference was detected between $gish^{MB896}$ heterozygotes and *ry*, or between $gish^{MB896}$ homozygotes and *ry* for either shock or odor avoidance. The concentrations used for olfactory conditioning in (A) and (B) were 0.6% for octanol and 0.25% for benzaldehyde.

ry background. The rut^{2080} allele has a P element inserted in the promoter region of the *rut* gene and is a strong hypomorph (Han et al., 1992). If *gish* function was dependent on the cAMP pathway, then rut^{2080}; $gish^{MB896}$/+ and rut^{2080}; $gish^{KG03891}$/+ flies would perform indistinguishably from rut^{2080} flies. However, we found that the presence of either *gish* mutant allele further reduced the performance scores of rut^{2080}. This reduction was close to additive (Figure 2A). To exclude the possibility that the additive effect was due to residual *rut* activity in the rut^{2080} allele, we employed the null allele rut^{1}. This allele carries a point mutation in the catalytic domain of the adenylyl cyclase and causes a complete loss of adenylyl cyclase activity (Levin et al., 1992). The *gish* mutant alleles further reduced the performance scores of rut^{1} flies (Figure 2B). These data indicated that *gish* functions outside of the cAMP signaling pathway defined by *rut*.

Gene Structure and Alleles of *gish*

The *gish* gene is located on chromosome 3R extending across ~30 kb of genomic DNA (Figure 3A). *Drosophila gish* is alternatively spliced like its closest relative in mammals, CKIγ3 (Zhai et al., 1995). Two major transcription initiation sites were predicted from the sequences of a collection of *gish* cDNA clones (Figure 3B). The *gish* mRNAs generated from the first transcription initiation site encode proteins with an extra 41 amino acids at the N terminus compared to those from the second transcription initiation site. Within the region encoding the kinase domain, two exons are utilized in a mutually exclusive way among *gish* isoforms (Figure 3B).

The $gish^{MB896}$ line carries an enhancer detector element in the second intron of the *gish* gene, upstream of the second transcription initiation site. Two additional alleles were available and characterized: $gish^{04895}$ and $gish^{KG03891}$. The $gish^{04895}$ and $gish^{KG03891}$ lines have P element insertions in the second and third exons of *gish*, respectively (Figure 3A). Both lines, like $gish^{MB896}$, exhibited male sterility. The $gish^{KG03891}$ allele also exhibited strong, but incomplete, recessive lethality.

To determine how *gish* expression was affected by these alleles, we designed three quantitative PCR amplicons for the *gish* gene: the "long" and "short" amplicons were specific for *gish* transcripts utilizing the first and second transcription initiation sites, respectively, while the "all" amplicon was common for all splice isoforms. We then measured the relative amounts of "long," "short," and "all" *gish* transcripts by quantitative RT-PCR in control and homozygous mutant heads (Figure 3C). *Gish* expression was altered in all three alleles. In $gish^{MB896}$, transcripts from the first but not the second initiation site were severely reduced in abundance. In $gish^{04895}$, transcripts from the first initiation site were almost undetectable while transcripts from the second initiation site were increased over control in abundance. Very little of the "long" transcripts and ~25% of the "short" transcripts were found in the $gish^{KG03891}$ homozygotes (Figure 3C). Over all, *gish* transcripts were downregulated about 50% in both $gish^{MB896}$ and $gish^{04895}$ homozygotes, while in $gish^{KG03891}$ homozygotes, less than 20% *gish* transcripts remained (Figure 3C).

We developed rabbit polyclonal antibodies against *Drosophila* Gish proteins. On western blots of head extracts, Gish antibodies recognized two major bands (Figure 3D). The

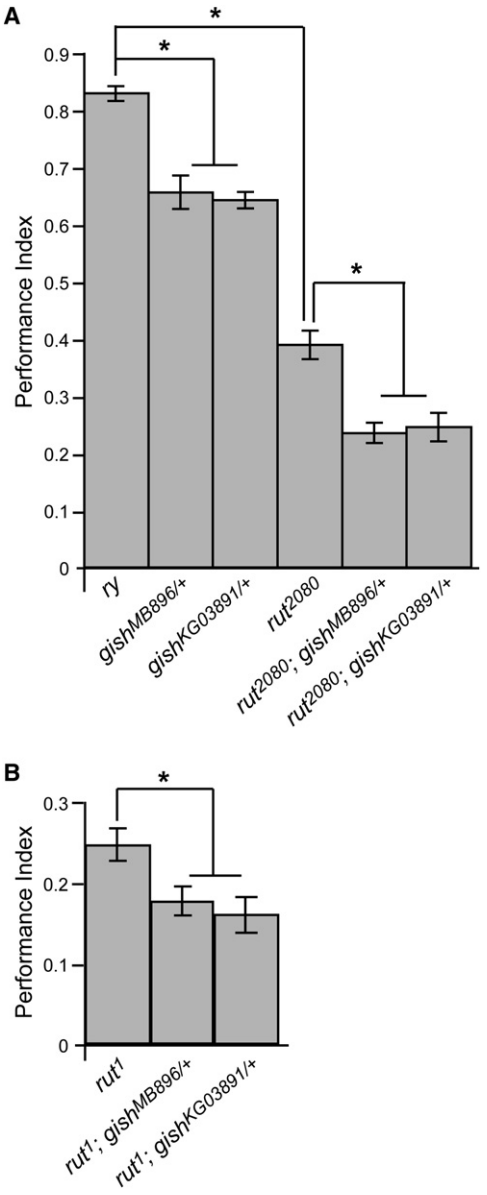

Figure 2. The Function of *gish* in Olfactory Learning Is Independent of *rutabaga*

(A) Epistasis analysis of rut^{2080} and *gish* alleles. The $gish^{MB896}$ and $gish^{KG03891}$ alleles were combined with rut^{2080} in the ry^{506} genetic background. Flies of the indicated genotypes were tested for 3 min memory. The performance scores of both *gish* alleles alone and rut^{2080} alone were significant lower than the *ry* control (Bonferroni-Dunn; $p < 0.0001$). The presence of either $gish^{MB896}$ or $gish^{KG03891}$ with rut^{2080} further reduced the performance score as compared to that of rut^{2080} alone (Bonferroni-Dunn; $p < 0.0001$). n = 12-16 for each group. (B) Epistatic analysis of rut^{1} and *gish* alleles. The $gish^{MB896}$ and $gish^{KG03891}$ alleles were combined with rut^{1} in the ry^{506} genetic background. Flies of the indicated genotypes were tested for 3 min memory. The performance of the rut^{1}; $gish^{MB896}$ or rut^{1}; $gish^{KG03891}$ was significantly different (*) from that of rut^{1} flies ($p = 0.0124$ and $p = 0.0022$, respectively, Bonferroni-Dunn). n = 23 for all groups.

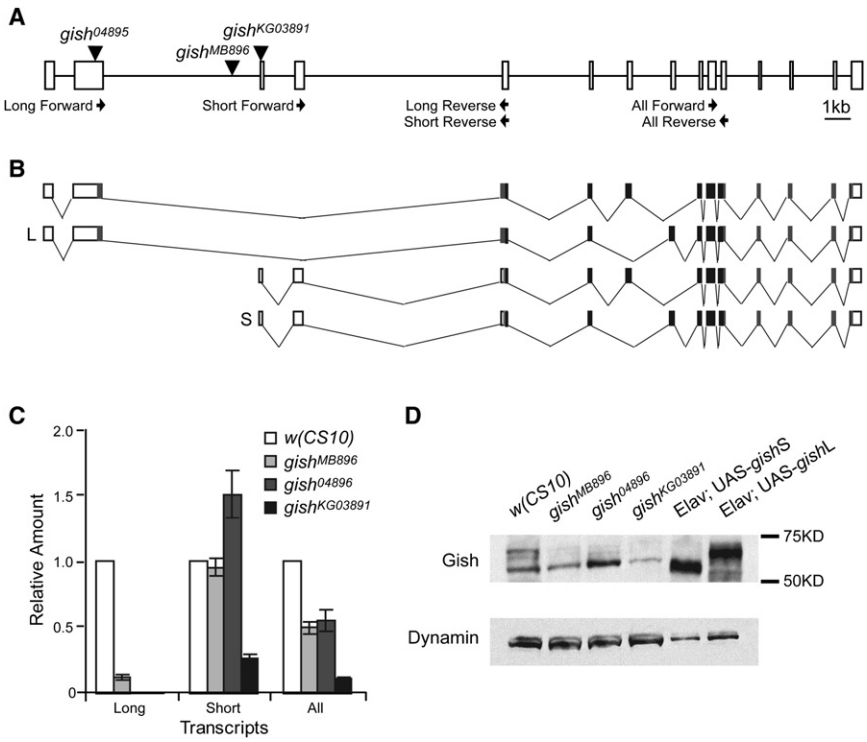

Figure 3. Gene Structure, Alleles, and Expression of *gish*

(A) P element insertion sites in the *gish* gene for various mutant alleles. Boxes indicate exons.

(B) Four major types of *gish* transcripts. Two transcription initiation sites, at exons 1 or 3, were predicted based on the sequences of available cDNA clones. Exons 7 and 8 were mutually exclusive among different transcripts. Grey and black shaded regions of the exons represent the open reading frame; the region encoding the kinase domain is shaded black. The two UAS-*gish* constructs used for overexpression correspond to the two major types of splice isoforms that utilized exon 8, labeled as L and S in (B).

(C) Altered expression of *gish* in different alleles. "Long" and "short" transcripts correspond to the transcripts that utilized the first and second transcription initiation sites, respectively. The relative amounts of "long," "short" or "all" *gish* transcripts in *gish* mutant lines were measured by quantitative RT-PCR. The sites of primers for long, short, and all transcripts QPCR are listed in (A).

(D) Western analyses of head extract from control, *gish* mutant, and *gish* overexpressing flies using anti-Gish antibodies. The total protein loaded for the overexpression lanes was 25% of that loaded for control and *gish* mutant lanes. The blot was reprobed with an anti-Dynamin antibody as the loading control.

correspondence of the two major protein bands to the two transcription units of *gish* was established by overexpressing UAS-*gish* transgenes in all neurons of the fly head using *Elav*-GAL4 and comparing the patterns in the head extract to that of controls. The high molecular weight band was diminished in all three *gish* mutants, while the low molecular weight band was diminished only in $gish^{KG03891}$ (Figure 3D). This result is consistent with the results of quantitative RT-PCR experiments and identifies the long and short transcripts as encoding the larger and smaller protein isoforms, respectively.

Memory Deficits in *gish* Mutants

We next examined the behavioral phenotypes of all *gish* alleles. All three *gish* alleles ($gish^{MB896}$, $gish^{04895}$, and $gish^{KG03891}$) were outcrossed to *w(CS10)* for 6 generations to normalize the genetic background. Memory retention in *gish* heterozygous mutants and *w(CS10)* control was tested at 3, 30, and 180 min after olfactory classical conditioning. The $gish^{KG03891}$ mutant as well as $gish^{MB896}$ performed more poorly than the *w(CS10)* control at all time points tested (Figure 4A). In the $gish^{04895}$ mutant, the memory deficits were evident at 30 min and 180 min but not at 3 min after training (Figure 4A). The less severe phenotype observed in $gish^{04895}$ compared to $gish^{MB896}$ and $gish^{KG03891}$ may be accounted for by functional compensation from the upregulation of *gish* "short" transcripts in $gish^{04895}$ (Figures 3C and 3D). Shock and odor avoidance for all three *gish* mutants were indistinguishable from the control (Figure 4B).

Expression Pattern of Gish in the Adult Brain

The nuclear *lacZ* reporter expression of $gish^{MB896}$ was detected in the photoreceptor cells, mushroom body cells, lamina, medulla, and local neurons around antennal lobes (Figures 5A and 5B). *Gish* expression in photoreceptor cells is consistent with its role in controlling glial cell migration in the developing eye (Hummel et al., 2002). To determine whether the enhancer detector in $gish^{MB896}$ reflected authentic *gish* expression, we performed in situ hybridization using an antisense probe against all *gish* splicing isoforms. The in situ signals detected in the neuronal perikarya confirmed the expression of *gish* in the mushroom bodies (Figures 5C and 5D). We further examined Gish protein localization by immunohistochemistry. Gish protein was found in the mushroom body calyces, peduncles and α, α', β, β', and γ lobes (Figures 5E–5H). Very little or no Gish protein was detected in mushroom body perikarya. We also observed significant Gish staining in the primary components of central complex, such as the ellipsoid body (not shown), the fan-shaped body (Figure 5G), the noduli (Figure 5G), and the protocerebral bridge (Figure 5H). Therefore, Gish is broadly expressed in the adult brain with an elevated expression in the mushroom body and central complex. The localization of Gish protein to the dendritic (calyces) and axonal (peduncles and lobes) compartments but not to the cell bodies (perikarya) of the mushroom bodies suggests that Gish functions in neuronal processes.

Partial Rescue of the Memory Deficit in *gish* Mutants

To prove that the memory deficit in *gish* was due to altered expression of the gene, we made a UAS-*gish* construct and generated independent transgenic lines to perform behavioral rescue experiments of the memory phenotype in *gish* mutants. The UAS-transgene carried a *gish* cDNA representing the first transcriptional unit and having two extra exons at the 3′ end

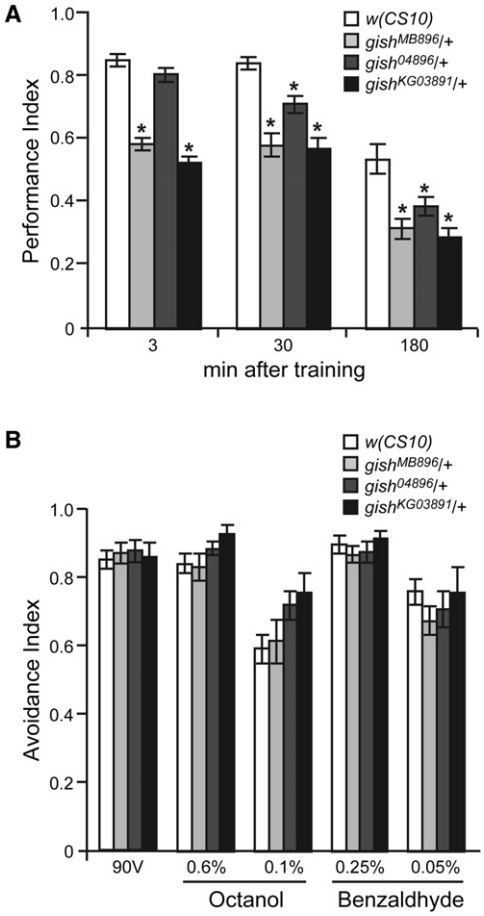

Figure 4. Memory Deficits in *gish* Heterozygous Mutants

(A) Memory retention in heterozygous *gish* mutants and *w(CS10)* control flies. Heterozygous *gish* mutants and *w(CS10)* were tested 3, 30, and 180 min after the olfactory classical conditioning. The performance of $gish^{MB896}$/+ and $gish^{KG03891}$/+ was significantly reduced compared to the control at all time points tested (Fisher's PLSD test; $p < 0.0001$). Significant differences between $gish^{04896}$/+ and control were detected at 30 and 180 min, (Fisher's PLSD test; $p = 0.0009$ and $p = 0.0006$ for 30 min and 180 min, respectively), but not at 3 min ($p = 0.2238$). n = 10–12 for each group.

(B) Odor and shock avoidance of *gish* mutants and control flies. The concentrations used for olfactory conditioning in (A) were 0.6% for octanol and 0.25% for benzaldehyde. No significant differences in avoidance indices were detected between *gish* mutants and *w(CS10)* control. n = 10 for each group.

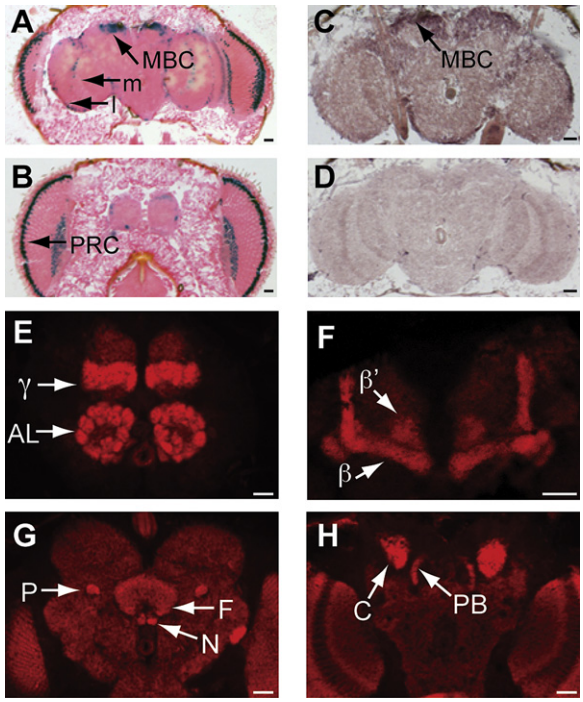

Figure 5. *Gish* Expression in Adult Fly Brain

(A and B) Expression of the nuclearly localized *lacZ* reporter in the frontal head sections of enhancer detection line, $gish^{MB896}$. Reporter expression was observed in mushroom body cells (MBC), photo receptor cells (PRC), and other brain regions such as lamina (l) and medulla (m). Scale bar in all panels: 20 μm.

(C and D) In situ hybridization with *gish* probes. Antisense (C) or sense (D) probe representing all splice variants of *gish* was hybridized to frontal sections of the adult brain. The *gish* mRNA was evident in the mushroom body cells (MBC).

(E–H) Immunohistochemistry with anti-Gish antibodies on cryosections of adult fly head. Gish protein was preferentially found in the calyces (C), peduncles (P), lobes of mushroom bodies (α/β, α′/β′, γ), antennal lobes (AL), fan-shaped body (F), noduli (N), and protocerebral bridge (PB).

(Figure 6A). The cDNA was recovered by RT-PCR using total RNA isolated from fly heads. The two extra 3′ exons indicated that there exist additional alternative splice forms for the *gish* gene.

Over several years of study, the dominant effect of *gish* alleles on memory formation in the *w(CS10)* background fluctuated while the memory deficits in homozygous *gish* mutants were more stable. Therefore, we decided to rescue the memory deficits in the $gish^{MB896}$ homozygous lines. The UAS-*gish* transgene was expressed in the mushroom bodies using the 247-GAL4 driver in homozygous $gish^{MB896}$ mutants (Figure 6B). This expression partially rescued the 3 min memory impairment of $gish^{MB896}$ homozygous mutants. These data map the requirement of 3 min performance on *gish* function to the expression domain of 247-GAL4. The 247-GAL4 line expresses in all three mushroom body lobes with significantly more levels found in the α/β and γ lobes than in α′/β′ (Schwaerzel et al., 2002; Krashes et al., 2007). A similar level of rescue was observed with 238Y-GAL4, another driver with expression in all types mushroom body neurons (see below). The failure to observe complete rescue could be due to insufficient levels of expression conferred by the GAL4 driver, a requirement for *gish* function outside of the expression domain of the driver, or a requirement for multiple isoforms of the *gish* gene. No rescue was observed if UAS-*gish* was expressed only in α′/β′ neurons by c305a-GAL4 or only in α/β neurons by c739-GAL4 in homozygous $gish^{MB896}$ mutants (Figure 6C).

Gish Plays a Physiological Role during Memory Formation

To determine whether the behavioral rescue observed was due to the expression of UAS-*gish* during development, adulthood,

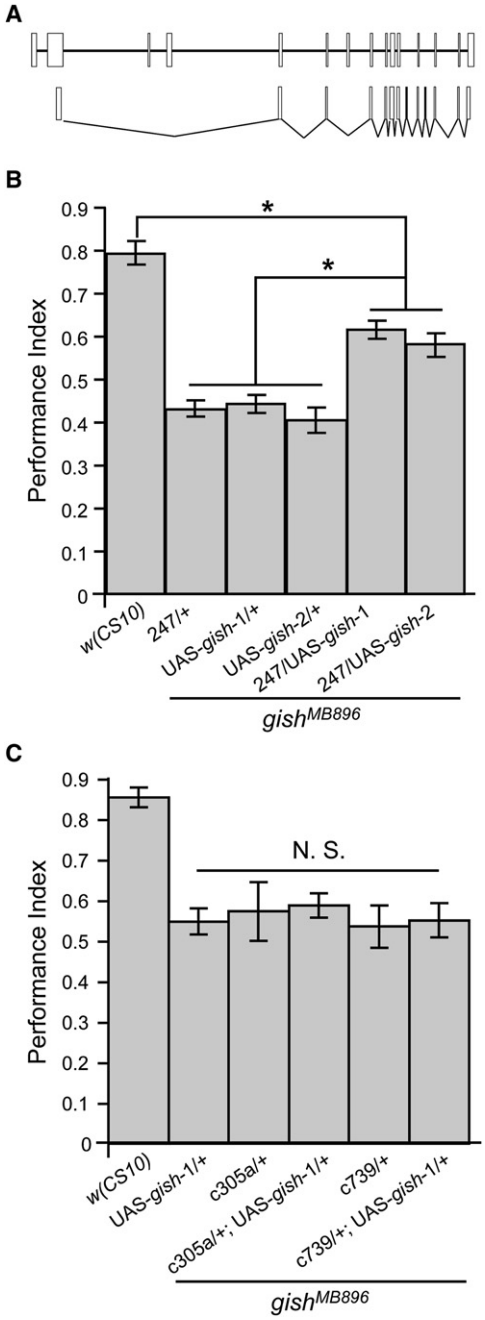

Figure 6. Partial Rescue of the Memory Deficits in *gish*^{MB896} Homozygotes with Expression of a UAS-*gish* Transgene in the Mushroom Bodies

(A) Exon composition of the UAS-*gish* transgene. Upper, *gish* genomic DNA; lower, a *gish* cDNA recovered by RT-PCR from head total RNA representing the *gish* "long" transcriptional unit. Two extra exons (red box) were identified in the 3′ of the *gish* gene with this cDNA. The cDNA was cloned into pUAST vector and transformed into flies.

(B) Partial rescue of the memory deficits in *gish*^{MB896} homozygotes with UAS-*gish* expression driven by 247-GAL4. Flies of the indicated genotypes were tested for memory retention at 3 min after olfactory associative training. The performance indices of flies carrying both 247-GAL4 and UAS-*gish* were significantly different from mutant controls (Bonferroni-Dunn; $p < 0.0001$), and also significantly different from the *w(CS10)* control (Bonferroni-Dunn; $p < 0.0001$) . n = 10-12 for each group. UAS-*gish*-1 and UAS-*gish*-2 represent two independent transgenes carrying the cDNA diagrammed in (A).

(C) No rescue of the memory deficits in *gish*^{MB896} flies with UAS-*gish* expression driven by c305a-GAL4 or c739-GAL4. The UAS-*gish*-1 transgene was expressed in the α′/β′ lobes by c305a-GAL4 or in the α/β lobes by c739-GAL4 in *gish*^{MB896} flies. Flies of the indicated genotypes were tested for memory retention at 3 min after olfactory associative training. No significant differences in performance indices were detected between any of the genotypes carrying the *gish*^{MB896} mutation. n = 6 for each group.

or both, we combined the TARGET system (McGuire et al., 2003) with 247-GAL4 and 238Y-GAL4 to drive UAS-*gish* expression in the mushroom bodies of *gish*^{MB896} mutants at different times of development. When flies were raised and maintained at 30°C, a temperature restrictive for the function of GAL80^{ts}, 247, or 238Y driven UAS-*gish* expression produced partial rescue of the *gish* learning impairment (Figure 7A), similar to that shown in Figure 6B. When flies were kept at 18°C throughout development and adulthood stages, a temperature permissive for the function of GAL80^{ts}, no rescue was observed (Figure 7B).

We next sought to determine whether expression of *gish* exclusively during the adult phase could rescue the memory impairment of *gish*^{MB896}. Flies of different genotypes were raised at 18°C until 1 day after eclosion and then shifted to 30°C for 4 days before testing. For both GAL80^{ts}/+; 247, *gish*^{MB896}/UAS-*gish*, *gish*^{MB896}, and GAL80^{ts}/+; 238Y, *gish*^{MB896}/UAS-*gish*, *gish*^{MB896} flies, expression of *gish* specifically in the adult phase produced significant rescue of the memory phenotype (Figure 7C). When UAS-*gish* was expressed only during development in *gish*^{MB896} mutant flies, no rescue was observed (Figure 7D). These results indicate that Gish participates in the physiological processes that underlie memory formation in adult flies, although they do not exclude an additional role in the development of the brain, since the rescue observed was incomplete.

Disruption of an Early Forming Memory Trace in *gish* Mutants

Behavioral memory is formed and stored through a set of changes, or memory traces, within nervous system that alter the processing and response to the sensory information that is learned. In the last few years, several different memory traces were discovered to form after olfactory associative training using functional optical imaging (Yu et al., 2004, 2005, 2006; Wang et al., 2008). One of these memory traces forms in the α′/β′ lobes of the mushroom bodies and is observed as enhanced Ca^{2+} influx in response to trained (CS^+) versus control odor (CS^-) after a single training trial (Wang et al., 2008). Since our results indicated that *gish* is required in the mushroom bodies for normal short-term memory formation, and this memory trace is the only one known to form within these cells shortly after training, we tested the hypothesis that *gish* function is required for the formation of the α′/β′ memory trace.

We used c305a-GAL4 to drive the expression of UAS-*G-CaMP*, a Ca^{2+} level indicator, in the α′/β′ lobes (Figure 8A). Groups of flies carrying c305a-GAL4 and UAS-*G-CaMP* were trained using octanol as CS^+ and benzaldehyde as CS^-, and then one fly was randomly selected from each group and

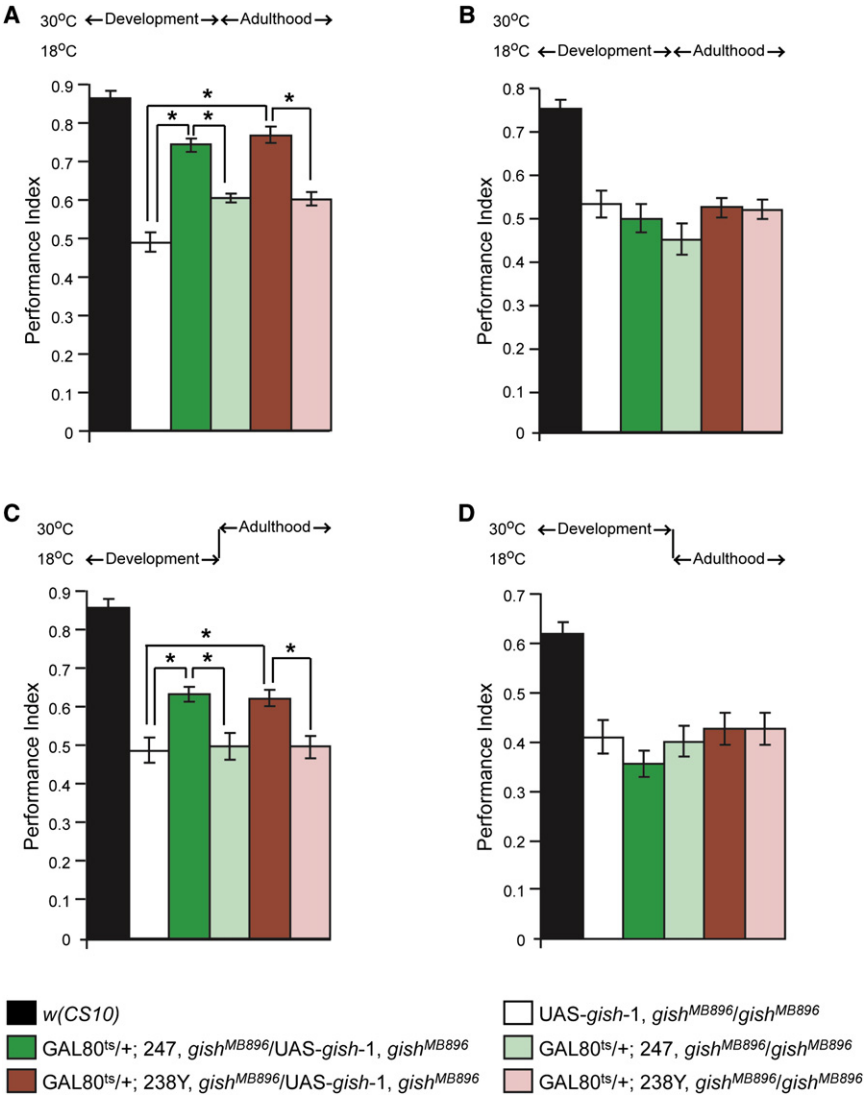

Figure 7. Conditional Rescue of the Memory Phenotype in *gish*^{MB896} Homozygotes using the TARGET System

(A) Partial rescue of the *gish* memory phenotype by the expression of *gish* throughout development and adulthood. A tubulin promoter-driven GAL80^{ts} transgene (denoted GAL80^{ts}) was combined with 247-GAL4 and 238Y-GAL4 to control the spatiotemporal expression of UAS-*gish*-1 in *gish*^{MB896} homozygotes. Flies were raised and maintained at 30°C to turn on UAS-*gish*-1 expression in mushroom bodies during both development and adulthood. The performance of GAL80^{ts}/+; 247-GAL4, *gish*^{MB896}/UAS-*gish*-1, *gish*^{MB896} flies was significantly increased over UAS-*gish*-1, *gish*^{MB896}/*gish*^{MB896} (Bonferroni-Dunn; $p < 0.0001$) and GAL80^{ts}/+; 247-GAL4, *gish*^{MB896}/*gish*^{MB896} (Bonferroni-Dunn; $p < 0.0001$) control flies. The performance of GAL80^{ts}/+; 238Y-GAL4, *gish*^{MB896}/UAS-*gish*-1, *gish*^{MB896} flies was also significantly increased over the UAS-*gish*-1, *gish*^{MB896}/*gish*^{MB896} (Bonferroni-Dunn; $p < 0.0001$) and GAL80^{ts}/+; 238Y-GAL4, *gish*^{MB896}/*gish*^{MB896} (Bonferroni-Dunn; $p < 0.0001$) control flies. Both GAL80^{ts}/+; 247-GAL4, *gish*^{MB896}/UAS-*gish*-1, *gish*^{MB896} flies and GAL80^{ts}/+; 238Y-GAL4, *gish*^{MB896}/UAS-*gish*-1, *gish*^{MB896} flies performed significantly worse than the *w(CS10)* control group (Bonferroni-Dunn; $p < 0.0001$ and $p = 0.0011$ respectively). n = 10 for each group.

(B) GAL80^{ts} repressed the GAL4/UAS-*gish*-1 rescue under permissive conditions. Flies were raised and maintained at 18°C throughout development and adulthood. n = 8 for each group.

(C) *Gish* expression in the mushroom bodies only during adulthood partially rescued the memory deficits. Flies were raised at 18°C and shifted to 30°C 1 day after eclosion. Flies were then maintained at 30°C for 4 days prior to training and testing. The GAL80^{ts}/+; 247-GAL4, *gish*^{MB896}/UAS-*gish*-1, *gish*^{MB896} flies performed significantly better than UAS-*gish*-1, *gish*^{MB896}/*gish*^{MB896} (Bonferroni-Dunn; $p = 0.0004$) and GAL80^{ts}/+; 247-GAL4, *gish*^{MB896}/*gish*^{MB896} (Bonferroni-Dunn; $p = 0.0009$) control flies. The performance of GAL80^{ts}/+; 238Y-GAL4, *gish*^{MB896}/UAS-*gish*-1, *gish*^{MB896} flies was also significantly better than the UAS-*gish*-1, *gish*^{MB896}/*gish*^{MB896} (Bonferroni-Dunn; $p = 0.0009$) and GAL80^{ts}/+; 238Y-GAL4, *gish*^{MB896}/*gish*^{MB896} (Bonferroni-Dunn; $p = 0.0020$) control flies. n = 10 for each group.

(D) *Gish* expression during development only was not sufficient for memory rescue in adults. Flies were raised at 30°C and shifted to 18°C 1 day after eclosion. Flies were then maintained at 18°C for 6 days prior to training and testing. n = 9 for each group.

prepared for live, functional imaging of Ca^{2+} responses in the mushroom bodies to the CS^+ and CS^- odors. The remainder of the flies in each group was tested behaviorally at 30 min after training, approximately the same time at which functional odor responses were recorded. Control flies trained with a forward protocol formed robust memory tested at 30 min (Figure 8B) along with a memory trace in the α'/β' lobes, quantified as the ratio between the response to the CS^+ and CS^- (Figure 8C). Control flies trained with a backward protocol failed to display behavioral memory or the memory trace at this time (Figures 8B and 8C). This early memory trace is best measured as the ratio of the response to the CS^+ and CS^-, consistent with prior results (Wang et al., 2008), because of large variation in the responses (Figure 8D). Importantly, *gish*^{MB896} mutants showed impaired 30 min memory (Figure 8B), as well as no detectable memory trace (Figure 8C). Similar effects of *gish* mutants on α'/β' memory trace formation were observed when flies were trained using benzaldehyde as CS^+ and octanol as CS^- (data not shown). Our data indicate that *gish* function is required for the encoding of both short-term memory and the α'/β' memory trace.

DISCUSSION

We provide evidence here, that *gilgamesh*, a CKIγ homolog, is required for short-term memory formation in *Drosophila* olfactory associative learning. We identified a *gish* mutant line, *gish*^{MB896},

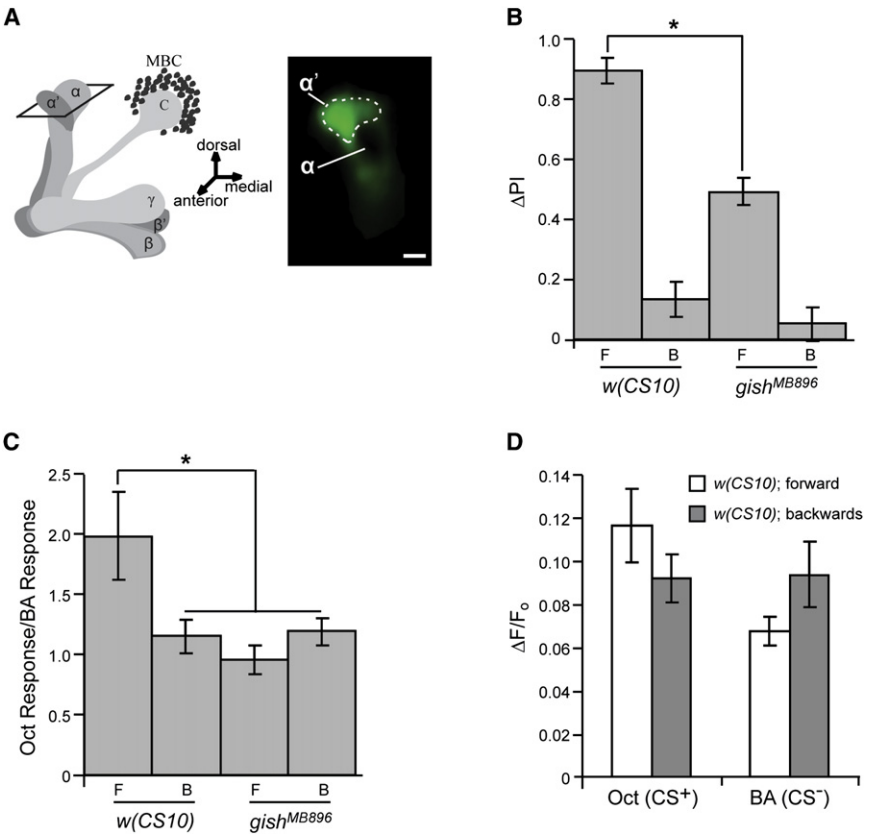

Figure 8. An Early Forming Memory Trace Is Disrupted in *gish* Mutants

(A) Left, an illustration of the structure of *Drosophila* mushroom bodies and the location from which functional images were obtained. Right, a representative image of the basal fluorescence in the tip of the α′ lobe for G-CaMP expressed under the control of c305a-GAL4. Fluorescence from the outlined area was used for quantification of G-CaMP response in the α′ lobe. C, calyx; MBC, mushroom body cells. Scale bar, 5 μm.

(B) Corresponding 30 min memory retention in trained flies. About 60 control or $gish^{MB896}$ homozygous flies carrying both c305a-GAL4 and UAS-*G-CaMP* were trained using either a forward or backward training protocol (CS^+: octanol; CS^-: benzaldehyde). One fly was then randomly selected for live, functional imaging (C and D) and the remainder was tested for memory retention at 30 min after training. Control flies receiving forward training performed significantly different from $gish^{MB896}$ flies receiving forward training (Fisher's PLSD test; $p < 0.0001$). F, forward trained. B, backward trained. In both (B) and (C), n = 16, 17, 12, and 12 for control forward, backward, $gish^{MB896}$ homozygous forward, and backward trained flies, respectively.

(C) Enhanced responses to the CS^+ relative to the CS^- odors in the α′ lobe in control flies receiving forward training, but not in $gish^{MB896}$ homozygous flies. The amplitudes of G-CaMP response were used to calculate the response ratio. The response ratio was significantly increased in forward trained control flies compared to any of the backward trained controls, forward trained $gish^{MB896}$ homozygous flies, and backward trained $gish^{MB896}$ homozygous flies (Fisher's PLSD test; $p = 0.0084$, 0.0031, and 0.0217, respectively).

(D) The amplitude of the G-CaMP response to octanol (CS^+) and benzaldehyde (CS^-) in the α′ lobe of control flies at 30 min after training. The trend for an increased response to the CS^+ and a decreased response to the CS^- in forward trained flies relative to backward trained flies failed to reach significance in group data due to significant variability. The ratio between these responses (C) offered the most reliable measure of this memory trace, consistent with prior results (Wang et al., 2008). n = 16 and 17 for forward and backward trained flies, respectively.

from a genetic screen for dominant effects on learning and memory in a collection of enhancer detector lines exhibiting preferential reporter gene expression in the mushroom bodies. In $gish^{MB896}$ mutants, performance was lower than the control at multiple time points tested after a single training trial. In addition, an early memory trace in the α′/β′ lobes was disrupted in $gish^{MB896}$ mutants, suggesting that *gish* function is required at the cellular level as well as the behavioral level for short-term memory formation. The expression of a UAS-*gish* transgene in the mushroom bodies only during adulthood partially rescued the memory deficits in $gish^{MB896}$, indicating that *gish* plays physiological role underlying learning and memory. In addition, we demonstrated with epistasis experiments that *gish*'s role in memory formation is independent of the cAMP pathway defined by *rutabaga* function.

A *rut*-Independent Pathway in Memory Formation in *Drosophila*

A long standing problem in *Drosophila* learning and memory has been to account for the 30%–50% (this study; Tully and Quinn, 1985) residual performance in the very strong alleles of *rut*. This residual memory indicates the existence of additional signaling pathways for memory formation. Many memory mutants have been identified, but none, until now, have been shown to disrupt the residual learning in strong *rut* mutants. Our genetic epistasis experiments demonstrated that *gish* alleles further reduce the performance score in severe *rut* mutants, indicating that *gish* functions independently of *rut*. Thus, gish appears to offer an entrée into a pathway for associative learning that is independent of *rut*. Other signaling pathways that may function in associative learning beyond those identified by *rut* and *gish* remain unknown. The essential nature of *gish* prohibits its complete removal to probe whether pathways other than those represented by *rut* and *gish* are required in the mushroom bodies for normal memory expression at early times after training.

A Protein Kinase Involved in Memory Formation

Since short-term memory is rapid, labile, and protein synthesis independent, posttranslational modifications are thought to be a major part of the biochemistry essential for this temporal form of memory. Many different protein kinases have been shown to have roles in memory formation, including protein kinase A, calcium:calmodulin-dependent protein kinase II, protein kinase C, mitogen-activated protein kinase, etc. (Micheau

and Riedel, 1999). This is the first report for a role of casein kinase I in memory formation. The specific form encoded by the *gish* mutant is casein kinase Iγ (CKIγ).

Unlike other protein kinases, CKI family members are constitutively active and insensitive to all known second messengers, like diacylglycerol, cAMP, and calcium (Tuazon and Traugh, 1991). As a constitutively active protein kinase, the expression level of CKI would be critical for its cellular function. Consistent with this characteristic, olfactory learning is sensitive to the expression level of *gish* since learning deficits are evident in heterozygous *gish* hypomorph alleles.

The CKI enzymes comprise a large family with a highly conserved kinase domain flanked by radically diverse amino and carboxyl termini (Gross and Anderson, 1998). In mammals, there are three CKIγ: γ1, γ2, and γ3. Recent in situ hybridization data from the Allen Brain Atlas indicates that the transcripts of all three CKIγ genes are expressed broadly in various brain regions. The similar expression patterns in brain suggest that these three mammalian CKIγ genes may have redundant neuronal functions.

Given the role of this enzyme in memory formation, the biologically relevant substrates of CKIγ, as well as the priming kinases for the substrates, assume importance but are yet to be identified. One candidate substrate is N-CAM. Mackie et al. (1989) reported the identification of two protein kinases from mammalian and avian brain that phosphorylated rodent and chicken N-CAM. Based on the chromatographic behavior and substrate specificity, the two kinases were identified as glycogen synthase kinase 3 (GSK-3) and CKI. Interestingly, the GSK-3 and CKI sites on N-CAM were phosphorylated only to a low level in vivo. It is possible that the GSK-3 and CKI sites may only be phosphorylated in response to specific stimuli in vivo. Furthermore, phosphorylation of N-CAM in vivo apparently occurs only when it is in or close to the surface membrane (Lyles et al., 1984). Among all of the CKI family members, only the CKIγs contain a putative prenylation site for membrane localization. This prenylation site is also present on Gish. When expressed in S2 cells, Gish is predominantly associated with the plasma membrane, while all other *Drosophila* CKIs are uniformly distributed in the cytoplasm (Zhang et al., 2006). N-CAM in vertebrates and apCAM in *Aplysia* have been suggested to be involved in memory formation (Murase and Schuman, 1999; Crossin and Krushel, 2000). FasII, the relative of N-CAM in flies, has been shown to be required in encoding short-term memories (Cheng et al., 2001). Thus, one emerging hypothesis is that CKIγ is involved in memory formation through phosphorylation of its substrate N-CAM/FasII in an activity-dependent manner.

Gish Mutants and the α′/β′ Cellular Memory Trace

Several different memory traces have been discovered to form in the olfactory nervous system after learning. One type studied here forms in the α′/β′ mushroom body neurons at early times (min) after conditioning. The trace emerges most prominently as a change in the ratio of calcium increases in these neurons in response to the CS^+ odor and the calcium decreases in response to the CS^- odor (Wang et al., 2008). Our studies reproduced the existence of this difficult to detect memory trace, and furthermore showed that *gish* function is required for the memory trace formation. Thus, *gish* is the first mutant identified to specifically disrupt the formation of this early memory trace along with early memory.

EXPERIMENTAL PROCEDURES

Fly Stocks and Genetics

Cantonized ry^{506} (*ry*) and w^{1118} [*w(CS10)*] were used as normal controls in our experiments. The $gish^{MB896}$ insertion was isolated in a screen for dominant effects on short-term memory among a collection of enhancer detector elements exhibiting preferential reporter gene expression in the mushroom bodies and recessive lethality or sterility. The $gish^{04895}$ and $gish^{KG03891}$ alleles were obtained from the Bloomington Stock Center. All three *gish* alleles were outcrossed to both *ry* and *w(CS10)* for six generations for behavioral experiments. PCR from single fly genomic DNA was performed to follow $gish^{MB896}$ and $gish^{04895}$ alleles in *w(CS10)*, and $gish^{KG03891}$ allele in *ry* in each generation of outcrosses. The primers used in the PCR are: $gish^{MB896}$ forward: 5′-TTG TGAGCGTGTGAAAATGC-3′; $gish^{04895}$ forward: 5′-TAGCACGAGGCTGTT TTCCT-3′; $gish^{KG03891}$ forward: 5′-GTTGTCATTCCGTC-3′; in combined with a common reverse primer: 5′-CTTGCCGACGGGACCACCTTATGTTA-3′, which was located in the P element.

Two independent transgenic lines, UAS-*gish*-1 and UAS-*gish*-2 were generated by transforming *w(CS10)* flies with the pUAST-vector containing a *gish* cDNA which utilizes the first transcriptional initiation site and has two extra exons in the 3′ region of the gene.

Molecular Biology

Primers and TaqMan probes for quantitative PCR were designed and synthesized by Applied Biosystems Assay-by-Design service. The sequences of the primers and probe for each amplicon were as follows:

CGAAATGCAGCGACGAGAAC (*gish* "long" forward primer); CCGGCAGT TGTCGTTTGTG (*gish* "long" reverse primer); CACCGCCACTTGCTTG (*gish* "long" probe);
CCCACTGAAAGTACCGTTCCA (*gish* "short" forward primer); CCGGCAG TTGTCGTTTGTG (*gish* "short" reverse primer); CCGCCACCGGTGGAC (*gish* "short" probe);
ATCGGTGATACGAAACGAGCAA (*gish* "all" forward primer); CAAACTC TTCCGGATGTCCATCA (*gish* "all" reverse primer); CCCATCGAGGTGC TTTG (*gish* "all" probe);
CACCAGTCGGATCGATATGCT (*rp49* forward primer); ACGCACTCTGTT GTCGATACC (*rp49* reverse primer); CATTTGTGCGACAGCTT (*rp49* probe).

Total RNA was isolated from fly heads using the TRIZOL reagent (Invitrogen) and reverse transcribed into cDNA using SuperScript III first-strand synthesis system (Invitrogen). Four independent cDNA samples of each genotype were prepared from four independent samples of total RNA. For each independent cDNA sample, quantitative PCR was performed in triplicate to measure *gish* "long," "short," "all," and *rp49* RNAs. The level of *gish* transcript in the mutants was first normalized to the loading control (*rp49*) and then to that of the *w(CS10)* control.

To generate anti-Gish polyclonal antibodies, a glutathione S-transferase (GST)-Gish (C-terminal 136 amino acids) fusion protein was expressed in *E. coli* BL21 using pGEX-KG vector. The purified fusion protein was injected into rabbits by Open Biosystems. The 1:1000 and 1:500 dilutions of anti-Gish anti-sera were used for western analysis and immunohistochemistry, respectively.

Behavior

Two- to four-day-old flies were used for all behavioral experiments except for the conditional rescue experiment. Training and testing were performed under dim red light at 25°C and 60% relative humidity using procedures described (Cheng et al., 2001). Briefly, flies were exposed to 1 min of an odor paired with 12 pulses of electric shock at 90 V (CS^+) followed by 1 min of a second odor without shock (CS^-). For 3 min memory

measurements, flies were immediately loaded into a testing maze and allowed to choose for 2 min between the CS^+ and CS^- odors. To assay memory retention at later time points, trained flies were transferred back into food vials for the appropriate interval and then tested as above. Except for the functional imaging experiments, two groups of flies were trained simultaneously using two different odors as the CS^+. The one-half performance index (PI) for each odor was calculated as: (number of flies that chose the CS^- minus the number that chose the CS^+)/(number of flies that chose the CS^- plus the number of flies that chose the CS^+). The overall PI was then calculated as the average of the two half PIs for each odor. For odor avoidance, untrained flies were loaded directly into a testing maze and allowed 2 min to choose between an odor of indicated concentration and air. For shock avoidance, both arms of the testing maze were replaced with shock tubes, and 90 V electric shocks were applied to one of the two arms for 2 min and the flies distributed between the arms according to their preference.

For functional imaging experiments, two different training protocols were used. The forward training protocol is described above. For backward training, the CS^+ odor was presented 45 s after the onset of the electric shock. A group of untrained flies of the corresponding genotype were tested for distribution in the testing maze in parallel. A distribution index was calculated for both the trained and untrained group and the ΔPI was obtained by subtracting the untrained index from the index of the corresponding trained group (Yu et al., 2006).

Functional Imaging

Functional imaging experiments were performed as described previously (Yu et al., 2006). After forward or backward training, flies were transferred into a new food vial. One fly was aspirated from the vial and mounted in a pipette tip. A small area of cuticle on the dorsal aspect of the fly head was removed and the opening covered with a small piece of plastic wrap. The flies were then mounted beneath a 20× objective lens of a Leica TCS confocal microscope and imaged using a 488 nm excitation laser line. The emitted light was collected from 520 ± 15 nm. Odorants were diluted in mineral oil and delivered from a micropipette in an air stream at a rate of 100 ml/min. The delivery of odors was under the control of a solenoid activated, three-way Teflon valve and a programmable controller, such that fresh air could be delivered to each animal for a predetermined period with an instantaneous switch to odor-laced air without altering the overall flow rate. The calcium response to the CS^+ (Oct) was assayed first by imaging with a 3 s odor exposure. After a 5 min interval, the calcium response to CS^- (Ben) was assayed in an identical way. Images were acquired at five frames per sec at a resolution of 256 X 256 pixels. Quantization of the responses was made from the pixels representing the dorsal tip of the α' lobe in each image. The F_o value was calculated for each pixel within the region of interest, as the fluorescence prior to odor application as averaged over five successive frames. The ΔF was calculated for each pixel as the difference between the highest average intensity during the 3 s odor application averaged over five successive frames and F_o.

Statistics

Data were analyzed with Statview. All data presented represent the mean ± the standard error of the mean. One-way ANOVA was followed by Bonferroni-Dunn or Fisher's PLSD analysis to test statistical significance.

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