GLD2 poly(A) polymerase is required for long-term memory

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The formation of long-term memory is believed to require translational control of localized mRNAs. In mammals, dendritic mRNAs are maintained in a repressed state and are activated upon repetitive stimulation. Several regulatory proteins required for translational control in early development are thought to be required for memory formation, suggesting similar molecular mechanisms. Here, using *Drosophila*, we identify the enzyme responsible for poly(A) elongation in the brain and demonstrate that its activity is required specifically for long-term memory. These findings provide strong evidence that cytoplasmic polyadenylation is critical for memory formation, and that GLD2 is the enzyme responsible.

In eukaryotic cells mRNAs are exquisitely regulated. mRNA stability, translation, and movement determine when, where, and how much protein each mRNA produces. Changes in poly(A) tail length can control translation and stability. Cytoplasmic lengthening of the tail can increase mRNA stability and translation, whereas shortening does the opposite. Activation of quiescent mRNAs, stored in a silent state in the early embryo, often requires the addition of poly(A) (1). Globally, mRNAs with longer poly(A) tails are translated more efficiently (1–3).

During early development, GLD2 poly(A) polymerase (PAP) is responsible for poly(A) tail lengthening and hence translational control (4–8). This divergent PAP adds poly(A) to specific mRNAs during meiotic maturation of oocytes in *Xenopus* and mice (4–6), as it does in the early *Drosophila* embryo (7) and the *Caenorhabditis elegans* germ line (8, 9). GLD2-related proteins, many with demonstrable polyadenylation activity, are conserved throughout evolution (8, 10). Whereas canonical nuclear PAP polyadenylates almost all mRNAs, GLD2 enzymes acquire substrate specificity by interacting with RNA-binding proteins and so are recruited to only a subset of mRNAs (4, 6, 8, 9). CPEB is such a specificity factor (11). PAPs more closely related to nuclear PAP also can participate in cytoplasmic events (8, 12).

Local control of mRNA translation is critical in synaptic plasticity and memory. In neurons, specific mRNAs are transported to dendrites in large RNP complexes called "neuronal granules." The granules contain repressed mRNAs and proteins involved in translational control and synaptic plasticity, such as Staufen, fragile X protein, Pumilio, and eIF4E (13, 14). Upon synaptic stimulation, previously quiescent dendritic mRNAs become translated. This activation is required for later phases of long-term potentiation, an electrophysiological correlate of long-term memory formation.

CPEB, which is present in dendrites, is required for memory in *Drosophila* (15) and long-term facilitation in *Aplysia* (16). CPEB has multiple molecular functions: it is involved in the transport, repression, and activation of specific mRNAs (11, 17). It is unclear which of these activities is essential for memory.

Here we identify in *Drosophila* a neuronal enzyme, GLD2, which possesses PAP activity. We show that it interacts with mRNA regulatory proteins, including dFMR and eIF4E, and co-localizes with these and other regulatory proteins in neuronal granules. We show that its enzymatic activity is essential for the formation of long-term memory, demonstrating that cytoplasmic polyadenylation is required for that process.

Results

DmGLD2 Is a Poly(A) Polymerase Localized in the Cytoplasm. To identify GLD-2–related proteins in *Drosophila* that have PAP activity, we created chimeras between MS2 coat protein and several *Drosophila* sequences related to GLD-2. Chimeric proteins were expressed in frog oocytes, in which the addition of poly(A) stimulates translation (Fig. 1*A*) (18). Two reporter mRNAs then were injected: a luciferase mRNA containing 3 MS2 binding sites and a β-galactosidase mRNA without these binding sites. An MS2 fusion with the *Drosophila* ORF, Dm1, most closely related to *C. elegans* GLD-2, enhanced translation of a luciferase reporter (Fig. 1*A*). The C-terminal portion of Dm1 (867–1360 aa), which includes the catalytic domain, was tested in the assay. An active site mutation (D991A) eliminated activation without affecting the abundance of the chimeric protein (Fig. 1*A*). The level of activation was similar to that of *C. elegans* GLD-2 (Fig. 1*A*). Consistent with these data, MS2-Dm1 protein lengthened a ^{32}P-labeled RNA that had MS2 binding sites, but an active site mutant form of Dm1 did not (Fig. 1*B*, *lanes 1–4*). The added nucleotides were poly(A), because they were removed by RNaseH/oligo(dT), and the RNA was bound oligo (dT) (Fig. 1*B*, *lanes 5–14*). We conclude that Dm1 is a poly(A)polymerase and refer to it here as "DmGLD2."

DmGLD2 RNA was expressed in the brain. A single mRNA species was detected in Northern blotting to whole-fly RNA, consistent with the analysis of cDNAs (Fig. 1*C*, *2 left gels* and *data not shown*). DmGLD2 mRNA was detected by RT-PCR throughout development including larvae, pupae, and adults. In adults, the mRNA was present in brain as well as the body (Fig. 1*C*, *rightmost gel*).

The regulated polyadenylation events implicated in memory and long-term potentiation are cytoplasmic. Two experiments demonstrate that DmGLD2 protein is localized to that compartment. FLAG-tagged full-length DmGLD2 expressed in *Drosophila* S2 cells was detected in cytoplasmic fractions (Fig. 1*D*). It also was detected in the nucleus and so mirrors the distribution of GLD2 protein in *Xenopus* oocytes (6). The effectiveness of the fractionation was corroborated using β-actin and histone proteins as markers (Fig. 1*D*). Similarly, a YFP– full-length DmGLD2 fusion protein expressed in fly motor neurons via

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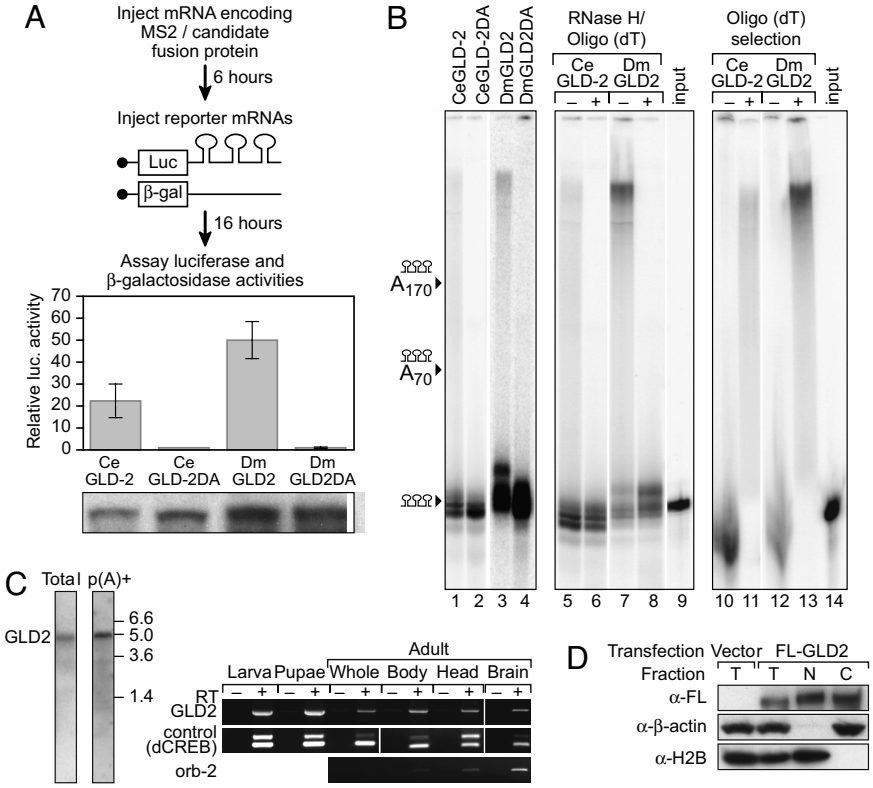

Fig. 1. Identification and characterization of *Drosophila* GLD2. (*A*) Tethered function assay. (*Top*) Strategy. *Xenopus* oocytes were injected with mRNAs encoding an MS2-GLD2 and incubated for 6 h. Luciferase mRNA containing 3 MS2 binding sites and a β-galactosidase mRNA without MS2 sites were co-injected. Luciferase and β-galactosidase activities were measured 16 h later. (*Middle*) Translation activity (luciferase normalized to β-galactosidase; CeGLD2DA set to 1.0). (*Bottom*) MS2-GLD2 fusion proteins detected by Western blot analysis with α-HA. (*B*) Dm1 has polyadenylation activity. ^{32}P-labeled RNA containing 3 MS2 sites was used as a substrate in polyadenylation assays in oocytes. Lanes 1–4: WT but not active site mutant (DA) DmGLD2 elongated ^{32}P-labeled RNA, as did CeGLD-2. Lanes 5–9: tails added by CeGLD-2 and Dm1 were destroyed by RNaseH/oligo(dT) treatment. −, no DNA oligo; +, with oligo(dT)$_{18}$. Lanes 10–14: RNAs bound to biotinylated oligo(dT) resin. −, RNA that did not bind; +, RNA that bound. Lanes 9 and 14: uninjected RNA substrates. Markers on the left indicate approximate tail lengths. The RNA carries 3 MS2 stem-loops. (*C*) DmGLD2 mRNA. The 2 left gels show Northern blots to total (T) and polyadenylated (pA+) RNAs from adult flies. Markers are shown to the right of the gels. The gel to the right shows DmGLD2 transcripts detected by RT-PCR. RNAs were prepared from larvae, pupae, and adults, and from adult bodies, heads, and the brain, as indicated above the lanes. Total RNA was reverse-transcribed with an oligo(dT)$_{18}$ primer, and the cDNAs were used as templates of PCRs to amplify DmGLD2 and the controls, dCREB and Orb2. dCREB control primers were expected to give 2 different bands because of alternative splicing. −, RT reactions without reverse transcriptase; +, RT reactions with the enzyme. (*D*) Subcellular distribution of DmGLD2 protein. FLAG-tagged DmGLD2 protein was expressed in S2 cells. Cells were lysed and fractionated into cytoplasm and nucleoplasm by centrifugation. DmGLD2 protein was detected by α-FLAG antibody. Actin and histone H2B monitor the fractionation, representing cytoplasm and nucleus.

UAS control was predominantly cytoplasmic but also was detected in nuclear puncta (see Fig. 3*A*).

DmGLD2 Interacts with eIF4E and dFMR1. The translation initiation factor, eIF4E, and the fragile X-mental retardation protein (FMRP/dFMR1) are both involved in memory formation (19, 20). To test whether these proteins interacted physically with DmGLD2, we performed co-immunoprecipitation experiments. α-DmGLD2 antibodies were incubated with crude fly extracts, and the bound proteins were detected by Western blotting. eIF4E and dFMR1 were co-immunoprecipitated with the DmGLD2 protein (Fig. 2*A*, *lanes 1–3*). Their interactions with DmGLD2 were RNA independent, because they were resistant to RNase treatment (Fig. 2*A*, *lanes 4–5*). Similar results were obtained using head extracts (data not shown). Interactions were confirmed using S2 cells expressing FLAG-tagged full-length DmGLD2. α-FLAG antibody immunoprecipitated eIF4E and dFMR1 from extracts of these cells (Fig. 2*B*). Neither protein was immunoprecipitated from S2 cells transfected with empty vector.

The interactions of eIF4E and dFMR1 with DmGLD2 were corroborated using recombinant GST-DmGLD2 fusion proteins. N-terminal (1–451 aa) or C-terminal (867–1360 aa) regions of DmGLD2 were purified from bacteria, attached to beads, and incubated with either *in vitro* translated proteins or crude fly extract. eIF4E bound N-terminal, but not C-terminal, DmGLD2 (Fig. 2*C upper bar*); dFMR1 interacted with both segments (Fig. 2*C lower bar*). All the interactions were RNA independent (Fig. 2*C*). Similarly, in fly extracts, eIF4E and dFMR1 bound immobilized GST-DmGLD2 (*data not shown*). We conclude that DmGLD2 physically interacts with eIF4E and dFMR1.

DmGLD2 Is Co-Localized with PUM and dFMR1 in Neuronal Granules. Several proteins involved in the regulation of mRNA translation and memory co-localize in mRNP complexes within dendrites (14). The composition of these mRNP complexes overlaps that of P-bodies and germ-line granules, both of which are associated with mRNA repression (13). To determine whether neuronal DmGLD2 was present in these particles, we created transgenic flies carrying a YFP–full-length DmGLD2 fusion under Gal4/UAS control and expressed the fusion protein in a subset of motor neurons. The distribution of YFP was analyzed in primary neuronal cultures.

YFP-DmGLD2 was found in the neuronal cytoplasm and in nuclear puncta (Fig. 3*A*). Additionally, YFP-DmGLD2 formed

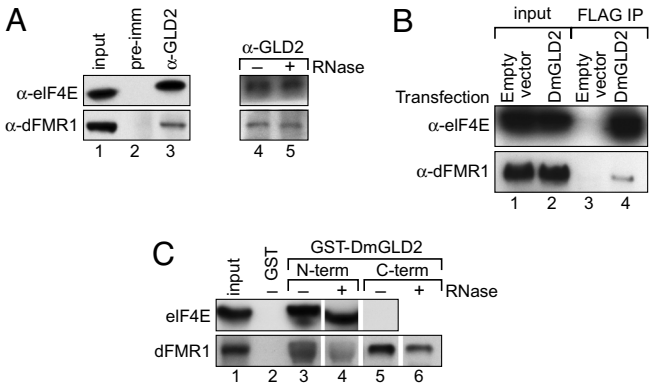

Fig. 2. DmGLD2 interacts with eIF4E and dFMR1 proteins. (*A*) eIF4E and dFMR1 interact with DmGLD2 in fly extracts. Crude fly extract was incubated with preimmune (lane 2) or α-DmGLD2 (lanes 3–5) sera. Lane 1: fly extract used in each immunoprecipitation (2%). eIF4E and dFMR1 proteins detected by Western blotting using α-eIF4E and α-dFMR1 antibodies, as indicated. −, RNase not added to reactions; +, RNase added. [RNAs were degraded by addition of RNase (data not shown)]. (*B*) Interactions in S2 cells. FLAG-tagged DmGLD2 or empty vector was transfected to S2 cells. FLAG-DmGLD2 expression was detected by Western blot analysis using α-FLAG antibody (see Fig. 1*D*). DmGLD2 was immunoprecipitated using α-FLAG antibody beads. Both eIF4E and dFMR1 were detected in lysates of DmGLD2-transfected cells (lane 4) but not of vector-transfected cells (lane 3). (*C*) Recombinant eIF4E and dFMR1 proteins bind GST-DmGLD2. GST-fusion of either N-terminal (1–451 aa) or C-terminal (867–1360 aa) DmGLD2 from *E. coli* was bound to glutathione agarose beads. *In vitro*–translated, HA-tagged eIF4E and dFMR1 were applied to beads carrying either GST (lane 2) or GST–GLD2 (lanes 3–6). eIF4E and dFMR1 were detected by Western blotting using α-HA antibody. Lane 1: *in vitro* translated proteins used in each GST pull-down (2%). All interactions were RNA-independent. −, RNase not added; +, RNase added.

discrete foci in neurites of cultured neurons, visualized with anti-HRP, a neuronal membrane marker (Fig. 3*B*). In neurites, YFP-DmGLD2 was present in mRNPs that contained dFMR1 (Fig. 3*C*) and Pumilio (Fig. 3*D*). Forty percent of YFP-DmGLD2 granules contained dFMR1 (n = 664 granules), and 63% contained Pumilio (n = 174 granules). The overlap is statistically significant and is consistent with the known compositional heterogeneity of Staufen/dFMR1 RNPs (13). We conclude that DmGLD2 is present in discrete neuronal particles associated with mRNA control.

Dominant-Negative DmGLD2 Inhibits Long-Term Memory Formation. NMDA-receptor–stimulated cytoplasmic polyadenylation is thought to be critical in localized translational activation in response to synaptic stimulation and hence for protein synthesis–dependent aspects of long-term potentiation (21). Our findings suggested that DmGLD2 might be the enzyme responsible for this polyadenylation. If so, learning or memory should be perturbed by disruptions of DmGLD2 activity. To test this hypothesis, we analyzed behavior in *Drosophila* expressing a point mutant form of DmGLD2 in which the active site of the enzyme was inactivated by a missense mutation. The analogous mutation in *Xenopus* GLD2 protein disrupts regulated polyadenylation in oocytes by titrating essential factors into inactive complexes, exerting a "dominant negative" effect (4).

We prepared transgenic flies bearing either the catalytically inactive, putative dominant-negative (DN) or active (WT) forms of DmGLD2 protein under the control of the heat-shock promoter. The transgenes carry the C-terminal 867-1360 aa of the protein, including the catalytic domain; we were unable to obtain full-length transgene expression under the heat-shock promoter control. The WT DmGLD2 possessed catalytic activity, but the mutant did not (Fig. 1). Two independent insertions were used

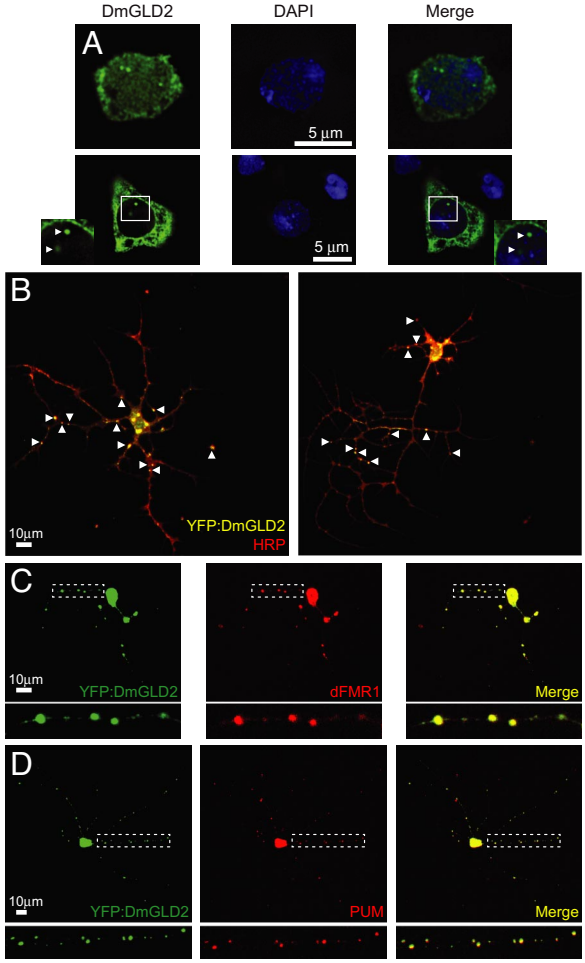

Fig. 3. DmGLD2 is in neuronal granules in neurites. (*A*) DmGLD2 is localized in cytoplasm and nucleoplasm. A single focal plane through the cell body of cultured motor neurons expressing YFP-DmGLD2 (green) counterstained with DAPI (blue). Boxed regions in lower left and lower right panels indicate areas shown in the magnifications. Arrowheads indicate discrete nuclear YFP-DmGLD2 foci, which often co-localize with dFMR1 and Pumilio (data not shown). (*B*) DmGLD2 in neurites. Confocal stack showing that YFP-DmGLD2 foci (merged in yellow) localize to neurites in cultured *Drosophila* motor neurons counterstained with an antibody against membrane-associated HRP (*red*). (Scale bar: 10 μm.) Arrowheads show positions of bright GLD2-containing foci. (*C* and *D*) DmGLD2 in mRNPs with dFMR1 and PUM proteins. Confocal stack showing neuritic YFP-DmGLD2 foci co-localize with endogenous (*C*) dFMR1-containing and (*D*) Pum-containing neuronal granules. Regions of high colocalization are indicated by dotted boxes and are shown below the main images. (Scale bar, 10 μm.)

for each transgene; all 4 lines expressed DmGLD2 protein upon induction (Fig. 4*A*, arrowhead), which was detected 4–13 h after heat shock.

We used 2 standard training regimens to quantify associative memory in flies. To measure immediate memory (by convention, termed "learning"), flies were exposed once to an odor that was paired with electric shock and then to a different odor that was not. This constituted a single training trial. The flies then were tested immediately for odor preference by placing them in the choice point of a T-maze, forcing them to choose between arms that contained the individual odors. Immediate memory (learning) dissipates rapidly (22). To measure long-term memory, flies received multiple training trials ("spaced training"), separated by rest intervals. This type of training produces protein synthesis–dependent memory that can persist for many days and is defined as long-term memory

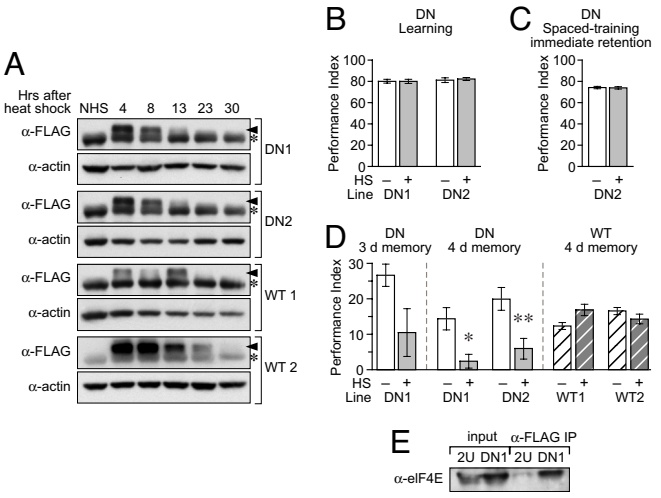

Fig. 4. DmGLD2 activity is required for long-term memory. In all cases, heat shock was for 30 min at 37°C. (*A*) Heat-shock induction profile of DN and WT gld2 transgenes. Transgenic flies carried FLAG-tagged active site mutant (D991A; DN) or WT DmGLD2 gene under the control of heat-shock promoter. Two independent lines of each transgene were used in this and all subsequent analyses (DN1 and DN2 and WT1 and WT2). GLD2 was measured by Western blotting with α-FLAG antibody; β-actin was a loading control. Arrowhead, FLAG-DmGLD2; asterisks, nonspecific background band; NHS, non–heat-shocked. (*B* and *C*) Dominant-negative (DN) DmGLD2 does not affect learning or immediate retention after spaced training. *gld2* Transgenes were induced by heat shock ending 3 h before training. Error bars indicate standard error of the mean, and asterisks indicate statistical significance (*, $P < 0.05$; **, $P < 0.01$) using ANOVA. (*B*) Neither DN-*gld2* transgenic line showed an induction-dependent effect on learning. (*C*) There was no induction-dependent effect on immediate performance after 10 cycles of spaced training. (*D*) DN gld2 transgene inhibits long-term memory. *gld2* Transgenes were induced by heat shock ending 3 h before training. Induced and uninduced flies were subjected to spaced training, and retention was measured at 3 and 4 days after training. For the DN transgene, both 3- and 4-day long-term memory was measured (solid bars); 4-day memory was measured for the WT transgenes (hatched bars). The DN transgenes inhibited formation of long-term memory (DN1 3-day, hs+ versus hs−, $P = 0.071$; DN1 4-day, hs+ versus hs−, $P = 0.017$l; DN2 at 4-day, hs+ versus hs−, $P = 0.0066$); the WT transgenes (hatched bars) did not. None of the other comparisons showed statistically significant differences. (*E*) Mutant DmGLD2DA binds eIF4E. WT (2U) flies and DN1 flies were heat shocked and recovered for 4 h. The WT flies (2U) carried no transgenes. Fly extract was applied to α-FLAG beads to perform immunoprecipitation. eIF4E was detected by Western blotting using α-eIF4E.

(23). To test the requirement for DmGLD2 in learning and long-term memory formation, 2 transgenic fly lines, DN1 and DN2, each carrying inactive DmGLD2 under control of the heat-shock promoter, were exposed to heat shock, or were not exposed, and then were allowed to recover for 3 h at room temperature, were trained behaviorally, and were tested for performance. As a control, behavior was assayed in flies carrying a WT version of the same transgene. Again, 2 fly lines were used, WT1 and WT2.

Induction of either line containing the catalytically inactive C-terminal partial DmGLD2 protein (DN) did not affect learning after a single training trial (Fig. 4*B*), nor did it affect memory measured immediately after spaced training (Fig. 4*C*). These data indicated that over-expression of inactive protein does not affect the ability of the flies to smell the odors, sense the shock, or move away from the inappropriate arm of the T-maze. In contrast, expression of the mutant DmGLD2 dramatically and specifically affected long-term memory, measured days after spaced training (Fig. 4*D*). Memory decreased substantially within 3 days after training and continued to decline thereafter (Fig. 4*D*).

The induced C-terminal partial DmGLD2 protein (DN) can interact with endogenous eIF4E protein in fly extracts (Fig. 4*E*), which suggests that it can interact with the normal partners of DmGLD2 and provides a likely explanation of its "dominant negative" mode of action. Disruption of long-term memory is highly specific: it was observed with both lines that expressed the catalytically inactive protein but in neither line that expressed its catalytically active counterpart. In addition, inhibition by the catalytically inactive allele depended entirely on heat-shock induction (Fig. 4*D*). The behavioral defects suggest that DmGLD2 enzymatic activity, presumably cytoplasmic poly(A) addition, is required for consolidation of long-term memories.

Discussion

The well characterized molecular mechanisms used in early development to regulate mRNAs are widely thought to be redeployed in somatic cells, including neurons. Localization of mRNA translation to active synapses involves several of the factors used in embryos and may provide the synaptic specificity that is required for memory formation (24). At present, 6 different mechanisms for translational activation have been proposed (25, 26). However, very few experiments examine the involvement of these mechanisms in a behaving animal. In this report, we provide evidence that an enzyme known to be critical in the regulation of mRNAs in the germ line also has a role in regulation at adult synapses *in vivo*. Our data support a model in which activity-regulated polyadenylation of synaptic mRNAs is required for long-term memory formation (15, 16, 21, 27).

The biochemistry and cell biology of DmGLD2 and its protein partners provide evidence for its role in synaptic regulation. DmGLD2 protein is cytoplasmic, interacts physically with eIF4E and dFMR1 proteins, and co-localizes in neurites to mRNPs that contain dFMR1, Pumilio, and other mRNA regulatory proteins (13). eIF4E, dFMR1, and Pumilio proteins function in the transport and translational control of mRNAs exported from the cell body (28). The mRNP puncta in neuronal processes are granules in which repressed mRNAs reside during transport (13). The presence of DmGLD2 in these repressive particles suggests that DmGLD2 may be regulated, perhaps through posttranslational modification or autoregulation of its own mRNA (29).

GLD2 is likely to be a pivot point in the regulation of dendritic mRNAs. Parallels between cytoplasmic polyadenylation in oocytes and synapses are striking: signals in the 3′UTR are interchangeable and recognized by the same protein, CPEB, which is activated in both cell types through phosphorylation by Aurora kinase. In the germ line, the consummation of the activation process is relief of repression by maskin (30) or 4E-T protein (31) and polyadenylation by the GLD2 PAP (4). Neuronal CPEB in *Drosophila* recently has been shown to play a role in long-term memory formation. Mutant flies carrying partial deletion of Orb2, a CPEB homolog expressed specifically in the brain, are defective in forming long-term memory (15). We propose that the PAP GLD-2 is an ultimate target of the Aurora kinase/Maskin/CPEB regulatory pathway and is acutely required during the period of long-term memory formation. As in oocytes, GLD2 probably functions with CPEB in controlling cytoplasmic polyadenylation at synapses; indeed, our preliminary results indicate that DmGLD2 and Orb2 co-immunoprecipitate (*data not shown*).

Our work relies on a DN, catalytically inactive allele of DmGLD2. Only the catalytically inactive allele perturbs memory, demonstrating the specificity of its effects on memory. The simplest interpretation of these findings is that disruption of memory requires titrated components to be sequestered in catalytically inactive complexes. Analysis of analogous mutations in the endogenous GLD2 locus will be instructive.

Despite clear data that polyadenylation occurs at synapses, before this work compelling evidence had not been presented

testing its role in memory in an animal. Molecular interpretation of the effects of CPEB disruptions are complicated by CPEB's multiple roles in repression, activation, polyadenylation, and mRNA localization. In the mammalian brain, GLD2 mRNA co-localizes with Pumilio and CPEB mRNAs, suggesting that GLD2 and its partners may all participate in similar neuronal events across species (6). Indeed, both CPEB/Orb2 and Pumilio are required for in memory formation (11, 32).

The demonstration that the GLD2 enzyme has a role in long-term memory provides new opportunities. The RNA substrates of GLD2-mediated activation in memory formation are likely to be mRNAs; their identification and validation is a critical next step, as is the use of loss-of-function mutants and perturbations of activity in specific regions of the brain. Effectors that modulate GLD2 activity are predicted to affect memory formation and so have potential clinical value. Elucidation of the molecular steps that regulate translation and of the ties between discrete molecular events and behavior is a major objective. The discovery of GLD2's role in memory will help achieve that goal and will bring studies of GLD2 regulation in the germ line to bear on that elusive mental process.

Methods

Tethered Function Assays. DmGLD2 (867–1,360 aa) (CG5732) and DmGLD2DA were cloned into pCSMS2 vector (6). CeGLD-2, CeGLD-2DA, luciferase, β-galactosidase, and small RNA reporter plasmids have been described (18), as have RNA preparation, injection, enzyme assays, and RNA analysis (18, 33).

Preparation of Antibodies and Immunoprecipitation. GST-DmGLD2 (867–1360 aa) was purified from *Escherichia coli* and injected into Guinea pigs. Crude serum (5 μl) was incubated with protein A beads for 1 h at 4°C and washed. Fly extract (500 μg), prepared by grinding flies in PBS, was added to the washed beads. After 4 h at 4°C, beads were washed 4 times with PBS containing 0.1% Nonidet P-40 and boiled in loading dye before SDS/PAGE. Western blots were probed with α-eIF4E antibody [1:1,000 dilution, gift from P. Lasko (McGill University, Montreal QC)] and α-dFMR$_{1800}$ antibody [1:500 dilution, gift from G. Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

GST Pull-Down. N-terminal (1–451 aa) and C-terminal DmGLD2 (867–1360 aa) were cloned into pGEX-6p vector (GE Healthcare Bioscience). GST-DmGLD2 from *E. coli* was bound to glutathione agarose beads (Amersham Bioscience). HA-tagged eIF4E and dFMR1 proteins were translated *in vitro* from PCR-generated templates using a rabbit reticulocyte-coupled T7 transcription and translation system (Promega). Pull-downs were performed in 20 mM Tris·HCl pH8, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA and 0.5% Nonidet P-40. Crude fly extract or *in vitro* translated proteins were added to GST-DmGLD2 beads and incubated for 2 h at 4°C. Beads were washed and boiled in loading dye for blotting.

Transfection and Immunoprecipitation. DmGLD2 bearing an N-terminal FLAG tag was cloned into pMtHy. The plasmid was transfected into S2 cells using Effectene reagent (Qiagen). Twenty-four hours later, protein expression was induced by the addition of 0.5 mM $CuSO_4$. Cells were incubated for another 24 h and harvested. Cells were lysed in 50 mM Tris·HCl, pH7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM PMSF, and protease inhibitors (RIPA buffer). α-FLAG antibody beads (Sigma) were washed with TBS, and 400 μg of cell lysate was added to beads. Binding was performed for 3 h at 4°C.

Transgenic Flies. DmGLD2 (867–1360 aa) with an N- terminal FLAG tag was inserted into pP(CaSpeRhs) (FlyBase), as was the active-site mutant form. Transgenic flies were prepared by injecting the plasmids into embryos (Bestgene). A heat-shock promoter drives transgene transcription; the polyadenylation region of Hsp70 flanks the insert. To express DmGLD2 proteins, flies were heat shocked at 37°C for 30 min and then allowed to recover at room temperature.

To generate flies expressing YFP-DmGLD2, DmGLD2 was cloned into the pTVW Gateway vector (The Drosophila Gateway Vector Collection, Carnegie Institution), and the plasmid was injected into embryos by Genetic Services, Inc.

Immunohistochemistry and Neuron Primary Cell Culture. Immunohistochemistry and primary cultures of *Drosophila* third instar larval thoracic-abdominal ganglia have been described (13). Briefly, a composite GAL4/Gal80 system (*D42-Gal4*; *chaGal80*) was used to drive expression of a YFP-DmGLD2 fusion protein in motor neurons. Cells were identified by the presence of YFP-DmGLD2–positive puncta allowing the identification of a discrete population of neurons. Primary antibodies used for YFP-DmGLD2 granule staining were rabbit α-GFP (1:200 dilution; Molecular Probes), mouse α-dFMR1 (6A15; 1:2500; Abcam) and rat α-Pum [1:1,000; a gift from R. Wharton (Duke University, Durham, NC)]. Secondary antibodies used were TRITC-conjugated goat α-HRP (Jackson ImmunoResearch), FITC-conjugated α-rabbit IgG (Sigma), and Alexa Fluor 568–conjugated α-mouse and α-rat IgG (Molecular Probes). Cultured cells were fixed, stained, and imaged on either an Olympus DeltaVision restoration microscope (Fig. 3*A*) or a Nikon PCM2000 laser confocal microscope (Fig. 3 *B–D*). Methods used to examine colocalization of dFMR1 and Pum to DmGLD2-containing puncta have been described previously (13).

Associative Learning and Memory Assay. We used the olfactory-avoidance conditioning paradigm of Tully and Quinn (34), modified to enable automated repetitive training regimens to assess learning and memory (35). A single-cycle of training consists of 90 sec exposure to ambient air; 60 sec of electric shock (the unconditioned stimulus); 70-V pulses lasting 1.5 sec and administered every 5 sec (12 total) accompanied by simultaneous exposure to 1 odor (the conditioned stimulus condition, CS+); 45 seconds of ambient air exposure to clear the first odor; 60 seconds of exposure to the second odor, with no shock (the CS− condition), 45 sec of ambient air to clear the second odor. Testing was done by placing flies in a choice point and allowing them to decide between the CS+ and CS− stimuli for 2 min. Learning was assayed directly after a single training cycle. Spaced training consists of 10 single cycles separated by 15-min rest intervals. We used 3-octanol and 4-methylcyclohexanol as odors. The performance index = [the number of flies making the correct choice] − [the number of flies making the incorrect choice]/total number of flies, multiplied by 100. To avoid odor-avoidance biases, we calculate the performance index of each single N by taking an average performance of 2 groups of flies, 1 trained with 3-octanol as CS+ , the other with 4-methylcyclohexanol. In all behavior experiments, $n = 8$.

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