Research

Elongator complex is required for long-term olfactory memory formation in *Drosophila*

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The evolutionarily conserved Elongator Complex associates with RNA polymerase II for transcriptional elongation. Elp3 is the catalytic subunit, contains histone acetyltransferase activity, and is associated with neurodegeneration in humans. Elp1 is a scaffolding subunit and when mutated causes familial dysautonomia. Here, we show that *elp3* and *elp1* are required for aversive long-term olfactory memory in *Drosophila*. RNAi knockdown of *elp3* in adult mushroom bodies impairs long-term memory (LTM) without affecting earlier forms of memory. RNAi knockdown with coexpression of *elp3* cDNA reverses the impairment. Similarly, RNAi knockdown of *elp1* impairs LTM and coexpression of *elp1* cDNA reverses this phenotype. The LTM deficit in *elp3* and *elp1* knockdown flies is accompanied by the abolishment of a LTM trace, which is registered as increased calcium influx in response to the CS+ odor in the α-branch of mushroom body neurons. Coexpression of *elp1* or *elp3* cDNA rescues the memory trace in parallel with LTM. These data show that the Elongator complex is required in adult mushroom body neurons for long-term behavioral memory and the associated long-term memory trace.

Introduction

The highly conserved hexameric Elongator complex was first characterized for its role in transcriptional elongation by its association with the RNA polymerase II holoenzyme (Otero et al. 1999). The *IKBKAP/elp1* gene encodes a scaffolding subunit of the complex and the *elp3* gene the catalytic subunit with histone acetyltransferase activity. Further studies have shown that the complex provides functions beyond histone acetylation (Wittschieben et al. 1999; Winkler et al. 2002), including acetylation of the cytoskeletal-like protein Bruchpilot (Miskiewicz et al. 2011), actin organization (Cheishvili et al. 2011; Jackson et al. 2014; Tielens et al. 2016), and the formation of modified wobble uridines in tRNA (Esberg et al. 2006). (for reviews, see Svejstrup 2007; Glatt and Müller 2013).

Mutations in Elongator subunits are associated with familial dysautonomia (FD), intellectual disability (ID), amyotrophic lateral sclerosis (ALS), and possibly rolandic epilepsy (RE) (Kojic and Wainwright 2016). A splice site mutation in human *IKBKAP/elp1* that skips exon 20 causes the severe neurodevelopmental disorder, FD, one of the most common hereditary sensory and autonomic neuropathies (Anderson et al. 2001; Slaugenhaupt et al. 2001). FD patients often fail to survive beyond 20 years of age (Axelrod 2004). The exon skipping varies in level across tissues and is especially severe in neurons leading to reduced IKBKAP/ELP1 in the central and peripheral nervous system (CNS and PNS) (Cuajungco et al. 2003; Boone et al. 2010). Mice null for *IKBKAP/elp1* do not survive beyond embryonic day 12.5; this lethality is rescued by expression of the human *IKBKAP* transgene (Chen et al. 2009). *Elp1* mutations in the mouse produce neuronal death in the PNS and abnormal development of the CNS (Jackson et al. 2014; Chaverra et al. 2017). Human genome association experiments have also linked Elp3 with ALS (Simpson et al. 2009; Kwee et al. 2012). Antisense morpholino knockdown of *elp3* in the zebrafish produces abnormal motor axons, a phenotype potentially related to ALS (Simpson et al. 2009). In addition, the Elongator complex has also been implicated in ID and possibly epilepsy. A deep sequencing study identified missense mutations in *elp2* associated with ID and related neurological disabilities. Recessive mutations in *elp2* were identified in three different families, each with members suffering from moderate or severe ID (Najmabadi et al. 2011; Cohen et al. 2015). An initial genome-wide linkage study associated *elp4* with RE (Strug et al. 2009), although this association has not been confirmed in subsequent studies (Gkampeta et al. 2014; Reinthaler et al. 2014). Nevertheless, the Elongator complex has multiple cellular roles and is involved in several different human disorders.

Drosophila melanogaster has been used as a model organism to study the basic neurobiology of olfactory learning and memory and human diseases for more than four decades (Heisenberg 2003; Davis 2005, 2011, 2015; Skoulakis and Grammenoudi 2006). Here, we probed the role of Elongator in olfactory memory formation. Given the embryonic lethality due to Elongator complex mutation in the mouse and the large body of evidence indicating important roles of the complex in the nervous system, we adopted two strategies for our studies. First, we used RNAi knockdown strategies rather than genomic mutations so that we could direct genetic insults to specific parts of the nervous system including the mushroom body neurons (MBn), neurons that have prominent roles in olfactory memory formation. Second, we used time and space conditional RNAi knockdown using Gene-Switch, a RU486-activatable *Gal4* that allows for transgene expression upon feeding flies the ligand RU486 (Mao et al. 2004; Tan et al. 2013). Our results show that two subunits of the Elongator complex, Elp1 and Elp3, are specifically required for protein-synthesis dependent aversive long-term memory (LTM), without roles in acquisition or short-term forms of olfactory memory. Moreover, the

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complex is required for the formation of a LTM molecular trace that forms in the MBn upon LTM formation.

Results

Elp3 is required for olfactory LTM generated by spaced conditioning

We searched and found a collection of RNAi transgenes from the VDRC *Drosophila* RNAi Center (Dietzl et al. 2007) against genes encoding the Elongator complex (Fig. 1G and Fig. 5G, below). The mushroom body Gene-Switch (MB-GS) *Gal4* line was used as the driver for expression of *uas*-RNAi transgenes to restrict expression of the RNAi's to the MBn (Mao et al. 2004). We constructed the flies so that they also carried *uas-dicer2* to increase RNAi efficacy. The expression of the *uas*-RNAi was induced by placing 1 d old adult flies on RU486-containing food for 3–4 d prior to training. Olfactory memory performance of this group of flies was compared to the same genotype kept on food without RU486, allowing for within genotype comparisons for potential roles of the individual RNAi transgenes on learning and memory. The administration of RU486 to control flies does not alter their memory performance (Mao et al. 2004).

Flies expressing *uas-elp3* RNAi 19470 ($elp3^{RNAi19470}$) in adult MBn exhibited a significant impairment in LTM tested at 24 h after five cycles of conditioning with a 15-min rest between cycles (spaced conditioning) relative to same genotype that remained uninduced using standard, two-odor classical conditioning (Fig. 1A). Two-odor classical conditioning involves exposing one group of flies to an odor CS+ along with an electric shock US followed by CS− odor exposure without shock prior to testing their preference for the CS− or CS+ in a T-maze. A second group of flies receives the same conditioning except that the CS+ and CS− odors are switched. The "half" Performance Indices for these two groups are then averaged to obtain a numerical index (PI) of their memory to the composite odor pair. No significant difference in performance between the RU486-fed and unfed control flies was detected at 3 min or 3 h after single cycle conditioning, or at 24 h after five cycles of massed conditioning (Fig. 1A). The impairment in LTM with expression of $elp3^{RNAi19470}$ in adult MBn was confirmed using single-odor spaced conditioning using OCT or BEN as the CS+ (Fig. 1B). Single-odor conditioning uses a "trained" group exposed to CS+/US pairing and CS−/no US during conditioning and testing against the CS+ and CS− odors in a T-maze. It also includes a "naïve" group of animals that undergoes mock training without exposure to any odors or electric shock. Performance Gains are calculated by subtracting the naïve group score to the CS+ odor from the score for the trained group. This protocol thus provides a numerical index of memory to a single odor rather than a mixed-odor pair. Our prior control experiments for single-odor conditioning using 1× backward, 5× massed backward, or 5× spaced backward training showed that these conditioning protocols failed to produce performance gains (Yu et al. 2006), arguing against a significant contribution of nonassociative factors to the Performance Gains obtained using forward conditioning protocols. Sensorimotor control experiments revealed that the *elp3* knockdown flies avoided odorants and electrified shock grids in ways indistinguishable from the control group (Fig. 1E). Thus, the deficit in LTM cannot be attributed to impairments at the sensory perception or motor performance levels.

We verified the behavioral phenotype of *elp3* RNAi knockdown using an independent *elp3* RNAi, $elp3^{RNAi106128}$, made against a different region of *elp3* mRNA (Fig. 1G). Flies expressing *uas-*$elp3^{RNAi106128}$ in adult MBn exhibited a significant impairment in 24 h LTM produced by two-odor, five cycle spaced conditioning relative to the uninduced control group (Fig. 1C). No differences were detected in 3 min or 3 h memory produced by single cycle conditioning, or in 24 h memory produced by five cycles of massed conditioning (Fig. 1C). The impairment in LTM produced by spaced conditioning with expression of $elp3^{RNAi106128}$ in adult MBn was further confirmed with single-odor conditioning using OCT or BEN as CS+ (Fig. 1D). No significant impairment was found in sensorimotor control experiments (Fig. 1F).

We examined the efficacy of knockdown in $elp3^{RNAi19470}$-expressing flies by Western blotting using a polyclonal anti-Elp3 antibody that we developed and by quantitative RT-PCR in heads, respectively. Western blotting revealed a reduction of ~60% in Elp3 protein content in RNAi expressing flies, supporting the specificity of the antibody (Fig. 2A). Quantitative RT-PCR experiments showed a reduction in the *elp3* mRNA of about 40% due to expression of this specific RNAi (Fig. 2B, left panel). Immunohistochemistry experiments showed that Elp3 protein is expressed broadly across brain neuropil (Fig. 3A). Quantitative immunohistochemistry focusing on the MBn revealed that expression of $elp3^{RNAi106128}$ in the MBn produced a reduction in signal of ~30% (Fig. 3B). Moreover, comparison of the staining pattern for anti-Elp3 compared to the nuclear marker anti-Elav indicated that Elp3 expression is largely cytoplasmic (Fig. 3C). Thus, our molecular analyses revealed a decreased expression of *elp3* mRNA and protein in the experimental genotypes, consistent with the hypothesis that decreased Elp3 activity, probably with a cytoplasmic function, produces the associated impairment in LTM (Fig. 1A,B).

We conclude from these experiments that the reduction of *elp3* mRNA and protein in the adult MBn has no effect on short-term, intermediate-term, or LTM produced by massed conditioning. Rather, the reduction specifically impairs protein-synthesis dependent LTM produced by multiple cycle, spaced conditioning.

Rescue of the LTM deficit by expressing an *Elp3* transgene in adult MBn

We generated a *uas-elp3* construct and subsequent transgenic lines to test the behavioral effects of Elp3 overexpression and to attempt behavioral rescue experiments. Two independent *uas-elp3* transgenic lines, *uas-*$elp3^{-1}$ and *uas-*$elp3^{-5}$, were selected. The two *elp3* transgenes were expressed in adult MBn using the MB-GS driver and feeding flies RU486. We observed no significant difference in the LTM of flies overexpressing *elp3* in adult MBn, indicating that the abundance of Elp3 is not limiting for LTM formation (Fig. 4A,B). Quantitative Western blotting experiments using an anti-Elp3 antibody revealed that expression of *uas-*$elp3^{-1}$ using a pan-neuronal driver produced a doubling of the Elp3 signal (Fig. 4G). Semi-quantitative immunohistochemistry experiments estimate the expression increase at ~150% of the control (Fig. 3B). These results indicated that overexpression of *elp3* in adult MBn does not affect LTM. However, flies coexpressing a *uas-elp3* transgene (*uas-*$elp3^{-1}$ or $elp3^{-5}$) along with *uas-*$elp3^{RNAi106128}$ in adult MBn showed control levels of 24 h LTM performance after single- and two-odor conditioning (Fig. 4C–E), indicating that coexpression of a wild-type *elp3* transgene rescues the LTM impairment associated with *uas-*$elp3^{RNAi106128}$ expression (Fig. 4C–E). These data make two important points. First, they show unambiguously that the LTM impairment associated with *elp3* RNAi expression is due to an insult on *elp3* mRNA since expressing a wild-type transgene reverses the phenotype. Second, they conclusively demonstrate that Elp3 function is required in the adult MBn for normal LTM conferred by spaced conditioning.

We next asked whether the LTM impairment due to *elp3* RNAi expression was reversible. Flies carrying $elp3^{RNAi106128}$ and *MB-GS* were fed on RU486 food for 4 d and then removed to normal food for five additional days. As expected, flies tested after 4 or 9 d of feeding on RU486 food exhibited the LTM impairment (Fig. 4F).

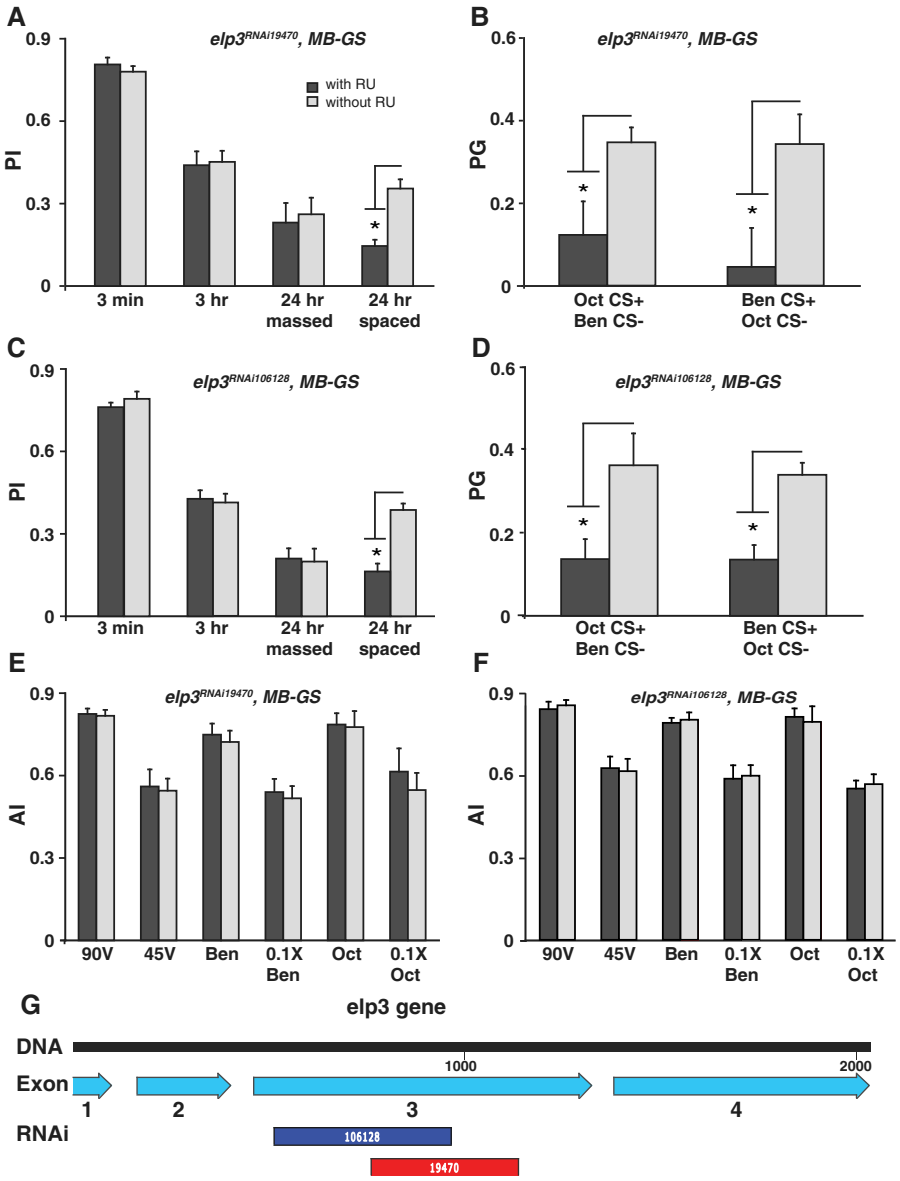

Figure 1. A LTM deficit associated with expression of *elp3* RNAi in the adult MBn. Performance index (PI) or performance gain (PG) of flies that had been fed for 3 d with or without RU486 before olfactory classical conditioning (*A–D*) or in shock and odor avoidance (AI) avoidance index experiments (*E, F*). Performance index (PI) is the average effect of conditioning using two different odors as CS+. Performance gain (PG) is the effect after single-odor conditioning. All flies carried the *Gal4* driver *MB-GS*, which provides for *Gal4* activity in the MBn only when RU486 is present. The flies also carried the indicated *uas*-RNAi transgenes and a *uas-dicer2 (dcr2)* transgene (not shown) to enhance the efficacy of RNAi knockdown. (*A*) Performance of flies expressing $elp3^{RNAi19470}$ in the adult MBn (genotype = $uas\text{-}elp3^{RNAi19470}$/+; *MB-GS, uas-dcr2*/+) after two-odor conditioning. Performance at 24 h after 5× spaced conditioning was significantly impaired by feeding RU486 (Mann–Whitney pairwise comparisons, $P < 0.0001$; $n = 8$ per group) but performance at 3 min or 3 h after 1× conditioning or at 24 h after 5× massed conditioning was not significantly different between the fed and unfed groups (Mann–Whitney pairwise comparisons, $P \geq 0.3124$; $n = 6$ per group). (*B*) Confirmation of the LTM deficit of flies expressing $elp3^{RNAi19470}$ in the adult MBn using single-odor conditioning. Performance at 24 h after 5× spaced conditioning with either OCT or BEN as the CS+ was significantly impaired by feeding RU486 (Mann–Whitney pairwise comparisons, $P \leq 0.0209$, $n = 6$ per group). (*C*) Performance of flies expressing $elp3^{RNAi106128}$ in the adult MBn (genotype = $uas\text{-}elp3^{RNAi106128}$/+; *MB-GS, uas-dcr2*/+) after two-odor conditioning. Performance at 24 h after 5× spaced conditioning was significantly impaired by feeding RU486 (Mann–Whitney pairwise comparisons, $P < 0.0001$; $n = 8$) but performance at 3 min or 3 h after 1× conditioning or at 24 h after 5× massed conditioning was not significantly different between the fed and unfed groups (Mann–Whitney pairwise comparisons, $P \geq 0.3138$; $n = 6$ per group). (*D*) Confirmation of the LTM deficit of flies expressing $elp3^{RNAi106128}$ in the adult MBn using single-odor conditioning. Performance at 24 h after 5× spaced conditioning with either OCT or BEN as the CS+ was significantly impaired by feeding RU486 (Mann–Whitney pairwise comparisons, $P \leq 0.0299$, $n = 6$ per group). (*E*) Shock and odor avoidance of flies expressing $elp3^{RNAi109470}$ in the adult MBn (genotype = $uas\text{-}elp3^{RNAi109470}$/+; *MB-GS, uas-dcr2*/+). Flies were challenged with a 90 V or 45 V shock versus no shock choice, or an odor (BEN or OCT) at the concentration used for learning experiments or at a 10-fold dilution (0.1×) versus a stream of fresh air and required to make a binary choice. No significant difference of shock and odor avoidance was detected between the fed and unfed groups (Mann–Whitney pairwise comparisons, $P \geq 0.5054$, $n = 8$ per group). (*F*) Shock and odor avoidance of flies expressing $elp3^{RNAi106128}$ in the adult MBn (genotype = $uas\text{-}elp3^{RNAi106128}$/+; *MB-GS, uas-dcr2*/+). Flies were challenged with a 90 V or 45 V shock versus no shock choice, or an odor (BEN or OCT) at the concentration used for learning experiments or at a 10-fold dilution (0.1×) versus a stream of fresh air and required to make a binary choice. No significant difference of shock and odor avoidance was detected between the fed and unfed groups (Mann–Whitney pairwise comparisons, $P \geq 0.6513$, $n = 8$ per group). (*G*) Exon organization *elp3* and location of RNAi sequences. The *elp3* gene is annotated with 4 exons. The $elp3^{RNAi106128}$ *and* $elp3^{RNAi19470}$ are directed against nonidentical but overlapping regions of exon 3.

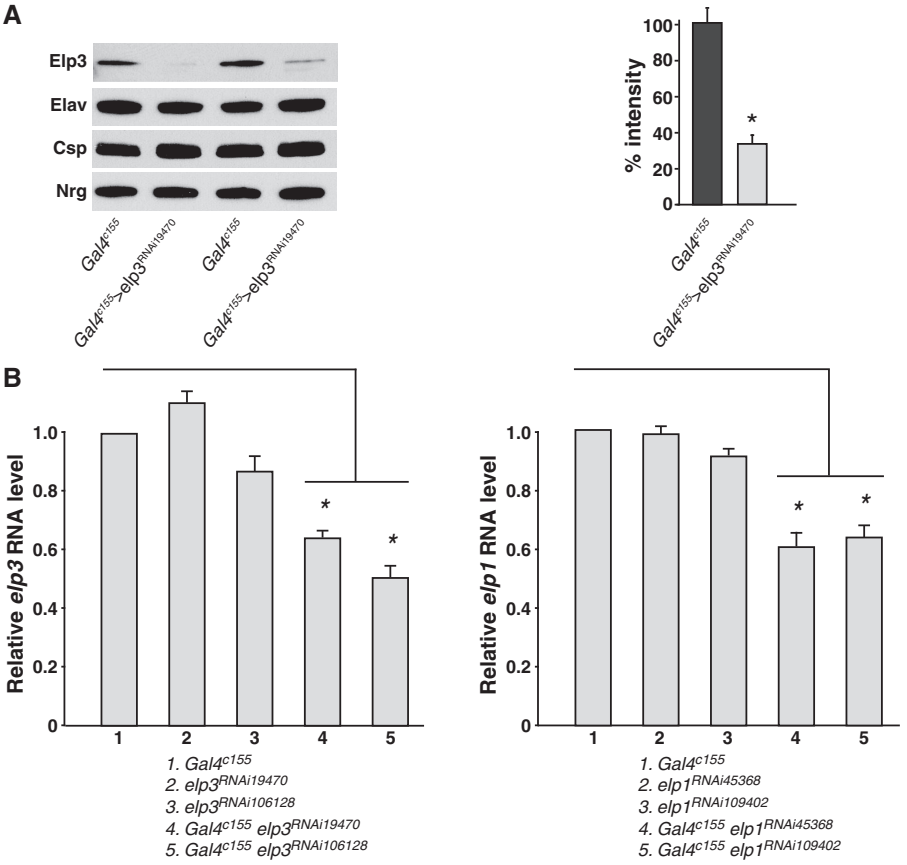

Figure 2. Efficacy of RNAi knockdown assayed by Western blotting and qRT-PCR. (*A*) Expression of Elp3 protein in *elp3* knockdown flies, $Gal4^{c155}$>$elp3^{RNAi19470}$ and controls. Western blots showing anti-Elp3 signal (~60 kD), anti-Elav (~52 kD), anti-Csp (~33 kD), and anti-Neuroglian (~180 kD), the latter three proteins as loading controls. Total protein extract from three fly heads was loaded in each lane, with eight independent samples ($n = 8$) for each genotype. The lanes for two control and two experimental genotype samples are shown. Quantification of Elp3 expression from the Western blots as a percentage of the control using the anti-Elp3 signal normalized to the anti-Elav signal for each genotype is shown at the *right*. Means ± SEM are shown. (*) $P < 0.0001$. (*B*) Quantification of Elongator complex mRNA in RNAi knockdown flies by qRT-PCR. Relative levels of *elp1* and *elp3* mRNAs in heads were measured by qRT-PCR normalized to *rp49* mRNA. (*Left*) *Elp3* mRNA in the brains of $Gal4^{c155}$/+, $elp3^{RNAi19470}$/+, $elp3^{RNAi106128}$/+, $Gal4^{c155}$> $elp3^{RNAi19470}$/+, and $Gal4^{c155}$> $elp3^{RNAi106128}$. The expression of *elp3* RNAi transgenes 106128 or 19470 using $Gal4^{c155}$ as a driver significantly decreased *elp3* mRNA levels compared to the $Gal4^{c155}$ or RNAi only ($elp3^{RNAi19470}$/+ and $elp3^{RNAi106128}$/+) controls. (Kruskal–Wallis multicomparison, $P = 0.0032$; Mann–Whitney pairwise comparisons, $P < 0.0001$, $n = 4$). (*Right*) *Elp1* mRNA in the brains of $Gal4^{c155}$/+, $elp1^{RNAi45369}$/+, $elp1^{RNAi109402}$/+, $Gal4^{c155}$> $elp1^{RNAi45369}$/+, and $Gal4^{c155}$>$elp1^{RNAi109402}$/+. The expression of *elp1* RNAi transgenes 45369 or 109402 using $Gal4^{c155}$ as a driver significantly decreased *elp1* mRNA levels compared to the $Gal4^{c155}$ or the RNAi only ($elp1^{RNAi45369}$/+ and $elp1^{RNAi109402}$/+) controls. (Kruskal–Wallis multicomparison, $P \leq 0.0016$; Mann–Whitney pairwise comparisons, $P < 0.0001$, $n = 4$).

However, flies withdrawn from RU486 food after 4 d and cultured for five additional days performed as well as those that had never experienced RU486 food. Thus, the Elp3 requirement for LTM was reversible, indicating a real-time requirement for normal level of this protein in MBn physiology for normal LTM.

Elp1 is required for olfactory LTM generated by spaced conditioning

Although the results above show that Elp3 is required for LTM generated by spaced conditioning, they do not address the question of whether this is due to Elp3 functioning in isolation or as part of the Elongator complex. Since Elp1 is a subunit of Elongator complex, we probed this issue by measuring the effect on memory formation of *elp1* RNAi expression in the adult MBn. Flies expressing either $uas\text{-}elp1^{RNAi109402}$ or $uas\text{-}elp1^{RNAi45369}$, which was made against different regions of *elp1* mRNA (Fig. 5G), in the adult MBn exhibited a significant impairment in 24 h spaced LTM relative to within genotype control flies after single- or two-odor conditioning protocols (Fig. 5A–D). No impairments were detected at 3 min or 3 h after single cycle conditioning or at 24 h after 5× massed conditioning (Fig. 5A, C). As with the RNAi knockdown experiments using *elp3*, the LTM impairments were not attributable to sensorimotor problems (Fig. 5E,F). Quantitative RT-PCR experiments indicated that these RNAi's were effective, decreasing *elp1* mRNA expression to ~60% of the control level (Fig. 2B, right panel). Thus, Elp1 along with Elp3 is required in adult MBn for LTM generated by spaced conditioning, consistent with the interpretation that the Elongator complex itself is essential.

We also performed overexpression and rescue experiments for *elp1* in ways identical to those described above for *elp3*. Wild-type transgenes ($elp1^{-6}$ and $elp1^{-5}$) were generated and used to overexpress Elp1 in the adult MBn. Single- and two-odor conditioning experiments revealed that such overexpression was without effect on 24 h memory generated by spaced conditioning (Fig. 6A,B), indicating that the abundance of Elp1 is not limiting for promoting LTM. Nevertheless, expressing either of these wild-type transgenes in adult MBn reversed the LTM deficit produced by expression of $elp1^{RNAi109402}$ and detected by single- or two-odor conditioning (Fig. 6C–E). These data, like those for *elp3*, conclusively show the requirement for Elp1 in adult MBn for LTM produced by spaced conditioning.

We tested the possibility that Elp1 and Elp3 provided redundant functions with phenotypic rescue experiments of the *elp3* RNAi LTM impairment by coexpression of wild-type *elp1*. The coexpression of the *uas-elp1* transgene $uas\text{-}elp1^{-6}$ and $uas\text{-}elp3^{RNAi106128}$ in the adult MBn using two-odor conditioning produced a LTM impairment like that observed with $elp3^{RNAi106128}$ expression alone (Fig. 6F, left panel). The reciprocal experiment was not performed. In addition, no sequence homology exists between the mRNA expressed from $uas\text{-}elp1^{-6}$ and the RNAi expressed from $uas\text{-}elp3^{RNAi106128}$. This result suggested Elongator complex function in LTM requires both *elp1* and *elp3*. We also attempted rescue of the $elp3^{RNAi106128}$ LTM phenotype with coexpression of a reported substrate of the Elongator complex (Creppe et al. 2009), *α-tubulin67c*. The coexpression of *uas-α-tubulin67c* (Venkei et al. 2006) and $elp3^{RNAi106128}$ in the adult MBn failed to rescue the LTM deficit due to $elp3^{RNAi106128}$ expression (Fig. 6F, right panel). This observation offers the possibility that α-tubulin67c may not be the sole downstream target of Elongator complex acetylation for normal LTM.

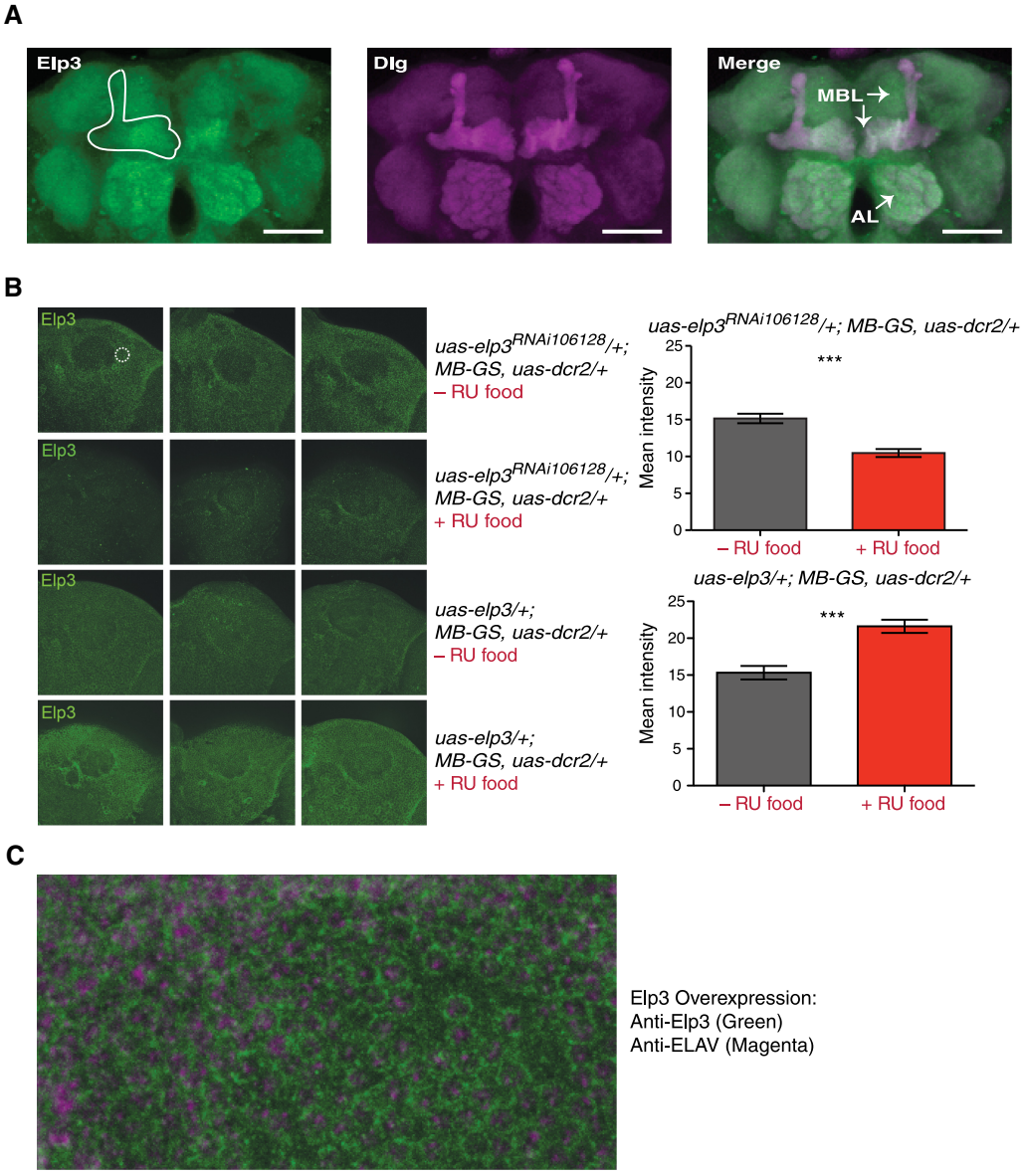

Figure 3. Expression pattern of Elp3 and immunohistochemical analyses of knockdown and overexpression. (*A*) Elp3 expression in the adult fly brain as detected by immunohistochemistry. Maximum projection images of the anterior brain immunostained with anti-Elp3 (*left*) and anti-Dlg (*middle*) antibodies. The *right* MB lobes are outlined in the *left* panel. (*Right*) Merged image from the *left* and *middle* panels. The anti-Dlg antibody highlights the MB lobes (MBL) and antennal lobe glomeruli (AL). Scale bar: 50 μm. (*B*) Immunohistochemical analyses of *elp3* knockdown and overexpression. The *left* set of images illustrate the anti-Elp3 staining in the area of the calyx for three representative flies carrying $elp3^{RNAi106128}$ raised on food without RU (first row), three flies carrying $elp3^{RNAi106128}$ raised on food with RU (second row), three flies carrying *uas-elp3* raised on food without RU (third row), and three flies carrying *uas-elp3* raised on food with RU (fourth row). A region of interest just lateral to the calyx (*top left* image, dotted line) was used to quantitate fluorescence across the four groups. The mean intensity of fluorescence in knockdown and overexpressing flies is shown in the bar graphs at the *right*. (Mann–Whitney pairwise comparisons: for $uas\text{-}elp3^{RNAi106128}$/+; *MB-GS, uas-dcr2*/+ between –RU food and +RU food, $P < 0.0001$, $n = 10$ flies per group. For *uas-elp3/+; MB-GS, uas-dcr2*/+between –RU food and +RU food, $P < 0.0001$, $n = 10$ per group. (C) Merged and magnified image of anti-Elp3 (green) and anti-ELAV (magenta) staining of neurons around the calyx of the MBs. Note that the anti-Elp3 staining largely encircles the nuclear-localized, anti-ELAV staining, indicating largely cytoplasmic expression.

Elongator function is required for the formation of an α-branch-specific LTM trace

Our prior experiments revealed that spaced conditioning generates a LTM molecular trace that forms in the α branch of the bifurcated axons of α/β MBn (Yu et al. 2006). This memory trace is detected as an increased calcium influx (increased GCaMP signal) in response to the presentation of the conditioned odor and forms between 3 and 9 h after spaced conditioning and persists for at least 24 h. This memory trace is tightly linked to behavioral LTM since both are dependent on normal protein synthesis, Creb function, Wnt signaling, and the activity of 26 other genes identified originally in screens for LTM mutants (Dubnau et al. 2003; Yu et al. 2006; Akalal et al. 2011; Tan et al. 2013). Our discovery that Elongator complex function is required for LTM posed the question of whether it is also required for the

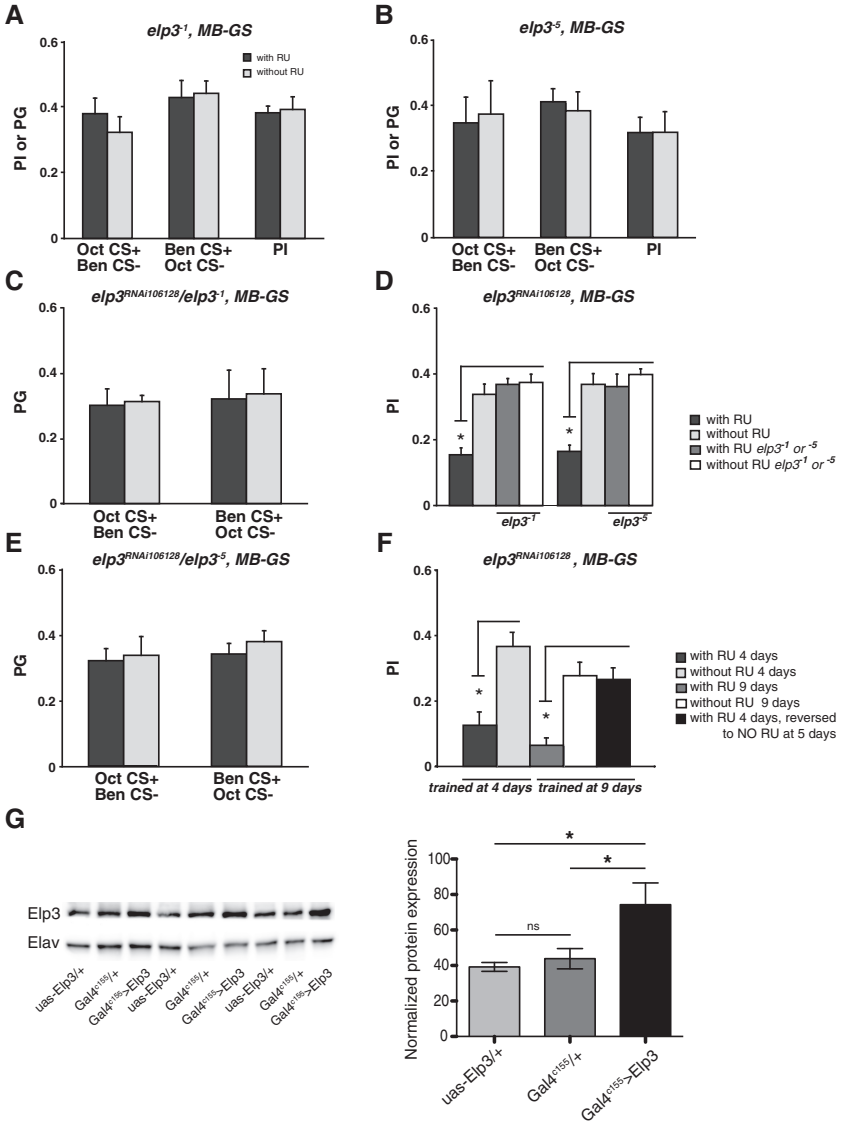

Figure 4. Rescue of the LTM impairment associated with *elp3* RNAi knockdown by overexpression of *uas-elp3* in the adult *Drosophila* MBn. Performance index (PI) is the average effect of conditioning using two different odors as CS+. Performance gain (PG) is the effect after single-odor conditioning. All flies carried the *Gal4* driver *MB-GS* and *uas-dicer2 (dcr2)*. Experimental data are within genotype, comparing the effects of being fed on RU486-laced food or on food without RU486. (*A*) Performance of flies expressing an *elp3* transgene (*uas-elp3*$^{-1}$/+; *MB-GS, uas-dcr2*/+) after single-odor or two-odor conditioning. Overexpression of *elp3* in the adult MBn had no significant effect on performance measured at 24 h after 5× spaced conditioning using single-odor (first two sets of bars) or two-odor protocols (third set of bars) (Mann–Whitney pairwise comparisons, P(0.2998, $n = 6$ per group)). (*B*) Performance of flies expressing an *elp3* transgene (*uas-elp3*$^{-5}$/+; *MB-GS, uas-dcr2*/+) after single-odor or two-odor conditioning. Overexpression of *elp3* in the adult MBn had no significant effect on performance measured at 24 h after 5× spaced conditioning using single-odor (first two sets of bars) or two-odor protocols (third set of bars) (Mann–Whitney pairwise comparisons, $P \geq 0.6024$, $n = 6$ per group). (*C*) Performance of flies coexpressing *elp3*^{RNAi106128} and wild-type *elp3*$^{-1}$ transgene in adult MBn (*uas-elp3*^{RNAi106128}/*uas-elp3*$^{-1}$*; MB-GS, uas-dcr2*/+) after single-odor conditioning. Flies expressing the RNAi along with the wild-type transgene (with RU) exhibited performance that was indistinguishable from flies in which transgene expression remained uninduced (without RU) (Mann–Whitney pairwise comparisons, $P \geq 0.7532$, $n = 6$ per group). (*D*) (*Left*) Performance of flies expressing only *elp3*^{RNAi106128} (*elp3*^{RNAi106128}/+; *MB-GS, uas-dcr2*/+) *or* coexpressing *elp3*^{RNAi106128} and wild-type *elp3*$^{-1}$ transgene in adult MBn (*uas-elp3*^{RNAi106128}/*uas-elp3*$^{-1}$*; MB-GS, uas-dcr2*/+) after two-odor conditioning. Flies expressing only *elp3*^{RNAi106128} in the MBn exhibited a significant decrement in performance compared to flies of the same genotype that remained uninduced (without RU) (Mann–Whitney pairwise comparisons, $P = 0.7261$, $n = 6$ per group). (*Right*) Performance of flies expressing only *elp3*^{RNAi106128} (*elp3*^{RNAi106128}/+; *MB-GS, uas-dcr2*/+) *or* coexpressing *elp3*^{RNAi106128} and wild-type *elp3*$^{-5}$ transgene in adult MBn (*uas-elp3*^{RNAi106128}/*uas-elp3*$^{-5}$*; MB-GS, uas-dcr2*/+) after two-odor conditioning. Flies expressing only *elp3*^{RNAi106128} in the MBn exhibited a significant decrement in performance compared to flies of the same genotype that remained uninduced and to other groups tested (Kruskal–Wallis multicomparison, $P = 0.0033$; Mann–Whitney pairwise comparisons, $P < 0.0001$, $n = 6$ per group). Flies expressing both *elp3*^{RNAi106128} and the wild-type *elp3* transgene in the adult MBn exhibited performance levels that were indistinguishable with flies in which transgene expression remained uninduced (without RU) (Mann–Whitney pairwise comparisons, $P = 0.4834$, $n = 6$ per group). (*E*) Performance of flies coexpressing *elp3*^{RNAi106128} and wild-type *elp3*$^{-5}$ transgene in adult MBn (*uas-elp3*^{RNAi106128}/*uas-elp3*$^{-5}$*; MB-GS, uas-dcr2*/+) after single-odor conditioning. Flies expressing the RNAi along with the wild-type transgene (with RU) exhibited performance that was indistinguishable from flies in which transgene expression remained uninduced (without RU) (Mann–Whitney pairwise comparisons, $P \geq 0.4978$, $n = 6$ per group). (*F*) Performance of flies expressing *elp3*^{RNAi106128} in adult MBn (*uas-elp3*^{RNAi106128}/+; *MB-GS, uas-dcr2*/+) after two-odor conditioning. Performance at 24 h after 5× spaced conditioning was significantly impaired by feeding RU486 for 4 d or 9 d (Kruskal–Wallis multicomparison, $P = 0.0005$; Mann–Whitney pairwise comparisons, $P \leq 0.0032$, $n = 6$ per group) compared with all other groups. Performance of flies with RU food 4 d and then removed to No RU486 food for 5 d was not significantly different compared with the unfed groups (Mann–Whitney pairwise comparisons, $P = 0.8076$; $n = 6$ per group). (*G*) Expression of Elp3 protein in *elp3* overexpressing flies, *Gal4*^{c155}*>elp3* and two controls. Western blots showing anti-Elp3 signal (~60 kD) and anti-Elav (~52 kD) staining, the latter protein as a loading control. Total protein extract from three fly heads was loaded in each lane, with six independent samples ($n = 6$) for each genotype. Three lanes for the two controls and the experimental genotype samples are shown. Quantification of *Elp3* expression as a percentage of the control using the anti-Elp3 signal normalized to the anti-Elav signal for each genotype is shown in the *right* panel (Kruskal–Wallis test, $P = 0.0106$; Dunn's multiple comparisons: *uas-elp3*/+ versus *Gal4*^{c155}/+: *ns*; *uas-elp3*/+ versus *Gal4*^{c155}*>elp3*: $P < 0.05$; *Gal4*^{c155}/+ versus *Gal4*^{c155}*>el*p3: $P < 0.05$).

formation of this LTM trace. *MB-GCaMP*, a transgene that expresses the GCaMP1.6 calcium reporter from a minimal heat-shock promoter under the control of the 247 bp MBn enhancer from the *Dmef2* gene was used to express GCaMP in the MBn independently of other transgenes under control of the *Gal4:uas* system (Tan et al. 2013).

Four groups of flies were tested for 24 h LTM after single-odor conditioning using OCT as CS+ or BEN as CS+ to investigate potential roles for *elp1* and *elp3* in the formation of the LTM trace. For *elp3*, these included flies expressing only *elp3*^{RNAi106128} (*elp3*^{RNAi106128}/+; *MB-GS, uas-dcr2/MB-GCaMP*) with and without RU486 administration, and flies coexpressing *elp3*^{RNAi106128}

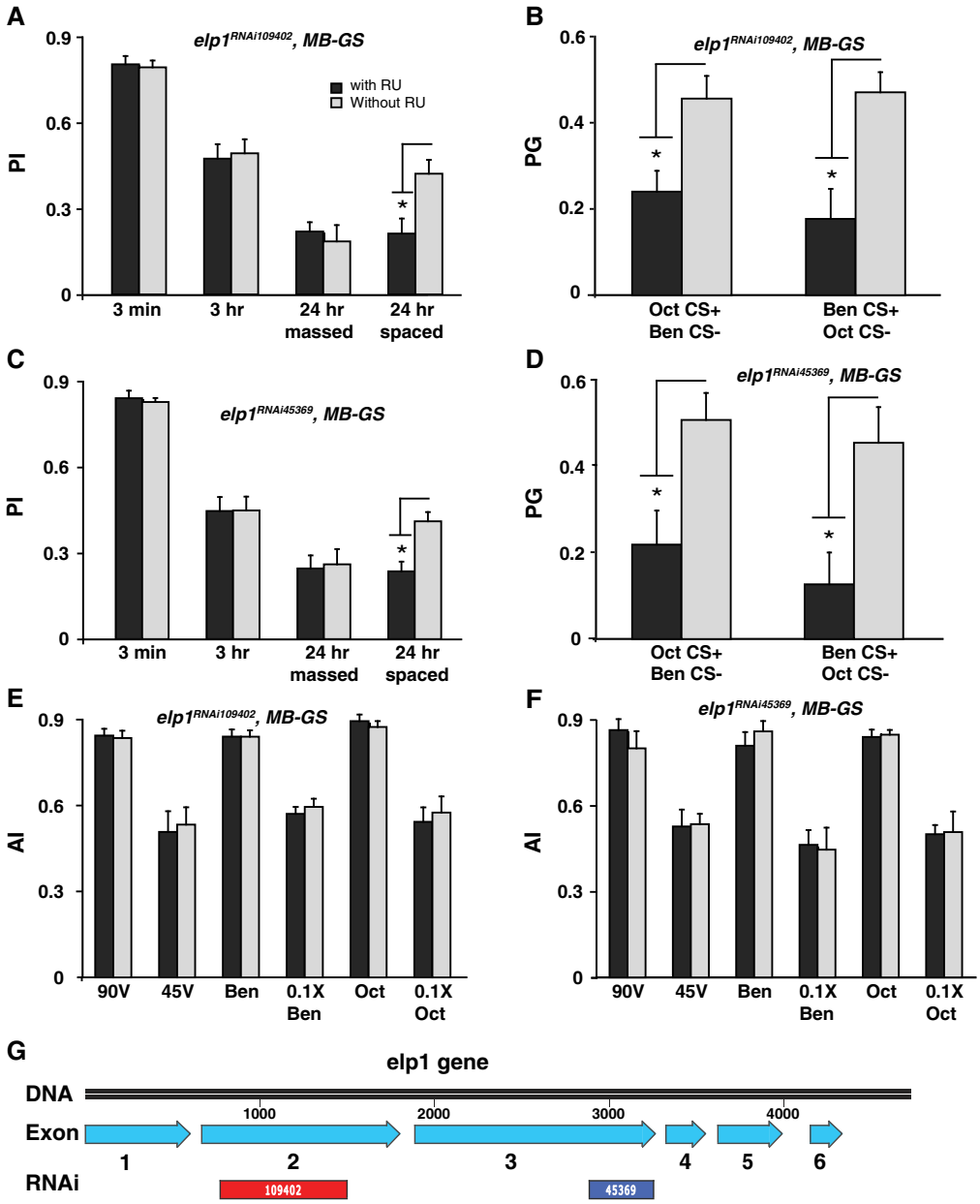

Figure 5. A LTM deficit associated with expression of *elp1* RNAi in the adult MBn. Performance index (PI) or performance gain (PG) of flies that had been fed with or without RU486 before olfactory classical conditioning (*A–D*) or in shock or odor avoidance (AI) avoidance index experiments (*E*, *F*). All flies carried the *Gal4* driver *MB-GS*, the indicated *uas*-RNAi transgenes, and a *uas-dicer2 (dcr2)* transgene (not shown). (*A*) Performance of flies expressing *elp1*^{RNAi109402} in the adult MBn (*uas-elp1*^{RNAi109402}*/+; MB-GS, uas-dcr2*/+) after two-odor conditioning. Performance at 24 h after 5× spaced conditioning was significantly impaired (Mann–Whitney pairwise comparisons, $P < 0.0001$; $n = 8$ per group) but performance at 3 min or 3 h after 1× conditioning or at 24 h after 5× massed conditioning was not significantly different (Mann–Whitney pairwise comparisons, $P \geq 0.2980$; $n = 6$ per group). (*B*) Confirmation of the LTM deficit of flies expressing *elp1*^{RNAi109402} in the adult MBn using single-odor conditioning. Performance at 24 h after 5× spaced conditioning with either OCT or BEN as the CS+ was significantly impaired by feeding RU486 (Mann–Whitney pairwise comparisons, $P \leq 0.0196$, $n = 6$ per group). (*C*) Performance of flies expressing *elp1*^{RNAi45369} in the adult MBs (*uas-elp1*^{RNAi45369}*/+; MB-GS, uas-dcr2*/+) after two-odor conditioning. Performance at 24 h after 5× spaced conditioning was significantly impaired (Mann–Whitney pairwise comparisons, $P < 0.0001$; $n = 8$ per group) but performance at 3 min or 3 h after 1× conditioning or at 24 h after 5× massed conditioning was not significantly different (Mann–Whitney pairwise comparisons, $P \geq 0.2859$; $n = 6$ per group). (*D*) Confirmation of the LTM deficit of flies expressing *elp1*^{RNAi45369} in the adult MBn using single-odor conditioning. Performance at 24 h after 5× spaced conditioning with either OCT or BEN as the CS+ was significantly impaired by feeding RU486 (Mann–Whitney pairwise comparisons, $P \leq 0.0258$, $n = 6$ per group). (*E*) Shock and odor avoidance of flies expressing *elp1*^{RNAi109402} in the adult MBn (*uas-elp1*^{RNAi109402}*/+; MB-GS, uas-dcr2*/+). Flies were challenged with a 90 V or 45 V shock versus no shock choice, or an odor (BEN or OCT) at the concentration used for learning experiments or at a 10-fold dilution (0.1×) versus a stream of fresh air and required to make a binary choice. No significant difference was detected between groups. (Mann–Whitney pairwise comparisons, $P \geq 0.6046$, $n = 8$ per group). (*F*) Shock and odor avoidance of flies expressing *elp1*^{RNAi45369} in the adult MBn (*uas-elp1*^{RNAi45369}*/+; MB-GS, uas-dcr2*/+). Flies were challenged with a 90 V or 45 V shock versus no shock choice, or an odor (BEN or OCT) at the concentration used for learning experiments or at a 10-fold dilution (0.1×) versus a stream of fresh air and required to make a binary choice. No significant difference was detected between groups (Mann–Whitney pairwise comparisons, $P \geq 0.5068$, $n = 8$ per group). (*G*) Exon organization of *elp1* and location of RNAi sequences. The *elp1* gene is annotated with 6 exons. The *elp1*^{RNAi45369} and *elp1*^{RNAi109402} RNAi sequences are directed against mRNA regions of exon 3 and exon 2, respectively.

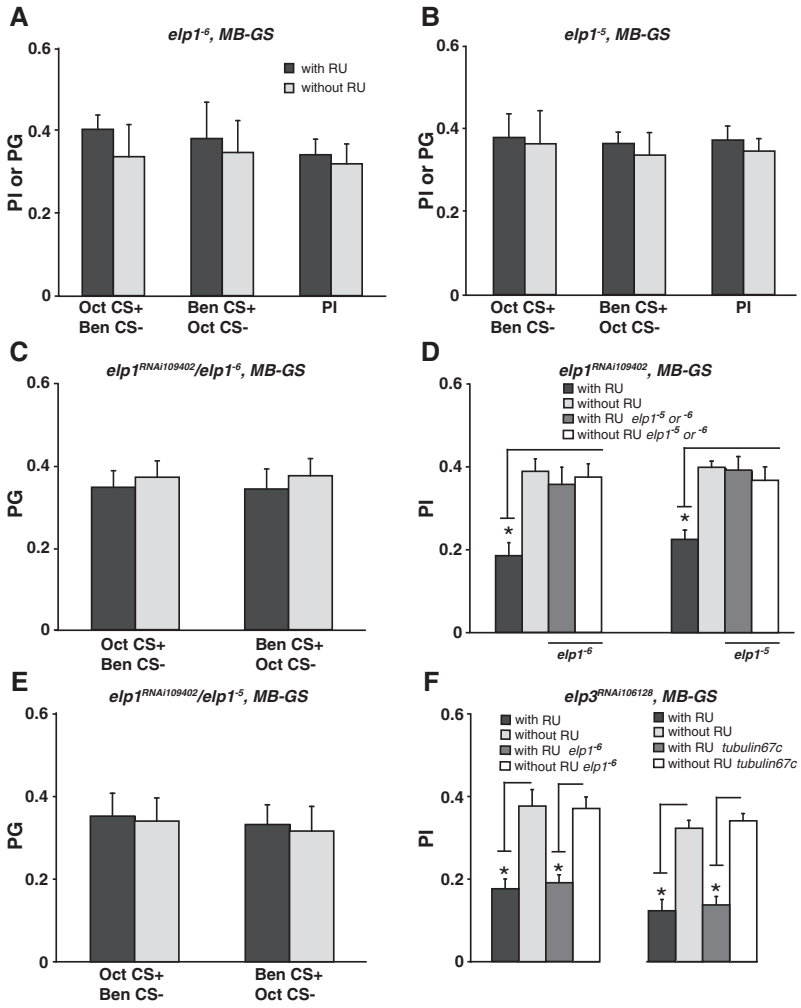

Figure 6. Rescue of the LTM impairment associated with *elp1* RNAi knockdown by overexpression of *uas-elp3* in the adult *Drosophila* MBn. Performance index (PI) is the average effect of conditioning using two different odors as CS+. Performance gain (PG) is the effect after single-odor conditioning. All flies carried the *Gal4* driver *MB-GS* and *uas-dicer2 (dcr2)*. Experimental data are within genotype, comparing the effects of being fed on RU486-laced food or on food without RU486. (*A*) Performance of flies expressing an *elp1* transgene (*uas-elp1*$^{-6}$*/+; MB-GS, uas-dcr2/+*) after single-odor or two-odor conditioning. Overexpression of *elp1* in the adult MBn had no significant effect on performance measured at 24 h after 5× spaced conditioning using single-odor (first two sets of bars) or two-odor protocols (third set of bars) (Mann–Whitney pairwise comparisons, $P \geq 0.2655$, $n = 6$ per group). (*B*) Performance of flies expressing an *elp1* transgene (*uas-elp1*$^{-5}$*/+; MB-GS, uas-dcr2/+*) after single-odor or two-odor conditioning. Overexpression of *elp1* in the adult MBn had no significant effect on performance measured at 24 h after 5× spaced conditioning using single-odor (first two sets of bars) or two-odor protocols (third set of bars) (Mann–Whitney pairwise comparisons, $P \geq 0.3345$, $n = 6$ per group). (*C*) Performance of flies coexpressing *elp1*^{RNAi109402} and the wild-type *elp1*$^{-6}$ transgene in adult MBn (*uas-elp1*^{RNAi109402}*/ uas-elp1*$^{-6}$*; MB-GS, uas-dcr2/+*) after single-odor conditioning. Flies expressing the *elp1* RNAi along with the wild-type *elp1* transgene (with RU) exhibited performance that was indistinguishable from flies in which transgene expression remained uninduced (without RU) (Mann–Whitney pairwise comparisons, $P \geq 0.3567$, $n = 6$ per group). (*D*) Performance of flies expressing only *elp1*^{RNAi109402} (*uas-elp1*^{RNAi109402}*/+; MB-GS, uas-dcr2/+*) *or* coexpressing *elp1*^{RNAi109402} and either the *elp1*$^{-5}$ or *elp1*$^{-6}$ wild-type transgene in adult MBn (*uas-elp1*^{RNAi109402}*/uas-elp1*$^{-5}$*; MB-GS, uas-dcr2/+, or uas-elp1*^{RNAi109402}*/uas-elp1*$^{-6}$*; MB-GS, uas-dcr2/+*) after two-odor conditioning. Flies expressing only *elp1*^{RNAi109402} in the MBn exhibited a significant decrement in performance compared to flies of the same genotype that remained uninduced and other groups tested (Kruskal–Wallis multicomparison, $P \leq 0.0068$; Mann–Whitney pairwise comparisons, $P < 0.0001$ for both sets of the four groups, $n = 6$ per group). Flies expressing both *elp1*^{RNAi109402} and the wild-type *elp1*$^{-6}$ in the adult MBn exhibited performance levels that were indistinguishable from the performance of flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P = 0.4234$, $n = 6$ per group). Flies expressing both *elp1*^{RNAi109402} and the wild-type *elp1*$^{-5}$ in the adult MBn exhibited performance levels that were indistinguishable from the performance of flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P = 0.4326$, $n = 6$ per group). (*E*) Performance of flies coexpressing *elp1*^{RNAi109402} and the wild-type *elp1*$^{-5}$ transgene in adult MBn (*uas-elp1*^{RNAi109402}*/uas-elp1*$^{-5}$*; MB-GS, uas-dcr2/+*) after single-odor conditioning. Flies expressing the *elp1* RNAi along with the wild-type *elp1* transgene exhibited performance that was indistinguishable from flies in which transgene expression remained uninduced (without RU) (Mann–Whitney pairwise comparisons, $P \geq 0.6523$, $n = 6$ per group). (*F*) (*Left*) Performance of flies expressing only *elp3*^{RNAi106128} (*uas-elp3*^{RNAi106128}*/+; MB-GS, uas-dcr2/+*) *or* coexpressing *elp3*^{RNAi106128} and the wild-type *elp1*$^{-6}$ transgene in adult MBn (*uas- elp3*^{RNAi106128}*/uas-elp1*$^{-6}$*; MB-GS, uas-dcr2/+*) after two-odor conditioning. Flies expressing only *elp3*^{RNAi106128} in the MBn exhibited a significant decrement in performance compared to flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P = 0.0001$, $n = 6$ per group). Flies expressing both *elp3*^{RNAi106128} and the wild-type *elp1*$^{-6}$ transgene in the adult MBn exhibited a significant decrement in performance compared to flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P = 0.0001$, $n = 6$ per group). Flies expressing the *elp3*^{RNAi106128} and flies expressing the RNAi along with the wild-type *elp1*$^{-6}$ transgene exhibited performance indices that were indistinguishable (Mann–Whitney pairwise comparisons, $P = 0.5321$, $n = 6$ per group; Kruskal–Wallis multicomparison, $P \leq 0.0006$, $P = 6$ per group). (*Right*) Performance of flies expressing only *elp3*^{RNAi106128} (*uas- elp3*^{RNAi106128}*/+; MB-GS, uas-dcr2/+*) *or* coexpressing *elp3*^{RNAi106128} and the wild-type α*-tublin67* transgene in adult MBn (*uas-elp3*^{RNAi106128}*/uas-α-tublin67; MB-GS, uas-dcr2/+*) after two-odor conditioning. Flies expressing only *elp3*^{RNAi106128} in the MBn exhibited a significant decrement in performance compared to flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P = 0.0001$, $n = 6$ per group). Flies expressing both *elp3*^{RNAi106128} and the wild-type α*-tublin67c* transgene in the adult MBn exhibited a significant decrement in performance compared to flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P = 0.0001$, $n = 6$ per group). Flies expressing the *elp3*^{RNAi106128} and flies expressing the RNAi along with the wild-type α*-tublin67c* transgene exhibited performance indices that were indistinguishable (Mann–Whitney pairwise comparisons, $P = 0.6523$, $n = 6$ per group).

and the wild-type *elp3*$^{-1}$ transgene in adult MBn (*uas-elp3*^{RNAi106128}*/uas-elp3*$^{-1}$*; MB-GS, uas-dcr2/MB-GCaMP*) with and without RU486 administration. The expression of GCaMP in MBn had no effect on LTM; the Performance Gains measured with these flies (inserts in Fig. 7A,C) were very similar to flies tested without expression of this calcium reporter (Figs. 1D, 4C). Flies expressing *elp3*^{RNAi106128} showed a LTM impairment that was rescued by coexpression of the wild-type *elp3*$^{-1}$ transgene after single-odor conditioning with OCT or BEN as CS+ (inserts in Fig. 7A,7C).

Some flies were removed from each of the trained groups shown in inserts in Figure 7A and C immediately before behavioral testing at 24 h after spaced conditioning, mounted for functional imaging, and tested for calcium responses to both the CS+ and CS− odors. We collected imaging data across time at the tip of the α branch of the α/β neurons. Flies expressing *elp3*^{RNAi106128}

showed a significantly reduced calcium influx in response to the CS+ when compared to the response of uninduced flies of the same genotype and flies coexpressing *elp1*^{RNAi109402} and wild-type *elp3*$^{-1}$ in adult MBn or flies of the same genotype that remained uninduced (Fig. 7A,C). The flies expressing *elp3*^{RNAi106128} elicited $\%\Delta F/F_o$ ratios to the CS+ odors that ranged from 4% to 6%. This response ratio is similar to odor responses obtained using naive flies, flies trained using protocols that fail to produce LTM (e.g., backward conditioning), and with CS– odors (Figure 7A,C; Yu et al. 2006). Importantly, *elp3*^{RNAi106128} expression did not affect the response to the CS– odor (Fig. 7B,D), showing that the RNAi expression specifically perturbs the memory trace. The other fly groups tested showed $\%\Delta F/F_o$ response ratios to CS+ odors of 9%–12% (Fig. 7A,C). This includes flies coexpressing *elp3*^{RNAi106128} and wild-type *elp3*$^{-1}$ in adult MBn and groups that were not fed RU486. These results reveal that the LTM memory trace impairment due to *elp3* RNAi expression in adult MBn is rescued with wild-type *elp3* expression.

Identical experiments were performed to test the role of *elp1* in LTM trace formation (Fig. 8). Expressing only *elp1*^{RNAi109402} (*uas-elp1*^{RNAi109402}/+; *MB-GS, uas-dcr2/MB-GCaMP*) in adult MBn impaired the formation of the CS+-specific LTM trace (Fig. 8A,C) with no detectable effect on the response to the CS– (Fig. 8B,D). This impairment was rescued by coexpression of wild-type *elp1*$^{-6}$ (Fig. 8A,C). In summary, formation of the α-branch-specific LTM trace requires the activity of the Elp3 and Elp1 subunits of the Elongator complex.

Discussion

Here, we demonstrate that Elongator complex function is required in adult MBn for normal olfactory LTM produced by spaced conditioning and an associated LTM memory trace, but not for short- or intermediate-term memory, or LTM produced by massed conditioning. We used both time and space conditional RNAi knockdown strategies for behavioral and functional imaging experiments, in conjunction with transgenic rescue experiments using wild-type transgenes, to make these discoveries. Our experimental design was "within genotype," with and without RU486 treatment. Prior studies have established that RU486-treatment itself is without behavioral effects on control genotypes (Mao et al. 2004; Tan et al. 2013; Qian et al. 2015). Recently, Chaverra et al. (2017) deleted *elp1* function throughout the nervous system of the mouse beginning at E11 to model the nervous system disruptions found in FD. The mutant mosaic mice exhibit a spectrum of phenotypes, including small size, unsteady gait, microcephaly, reduced motor neuron number, CNS neurodegeneration, reduced anxiety, and impairment in a long-term form of spatial memory. The latter phenotype may be most closely aligned with our observation of impaired LTM in *elp3* or *elp1* knockdown flies. The more restrictive phenotypes observed here beyond possible differences in function between model organisms are likely due to our specific knockdown in the MBn and in the adult stage of the organism. This strategy bypasses phenotypes of developmental origin.

The major issue for the future concerns where and how Elongator complex functions in the MBn for its role in LTM. Elongator complex involvement in post-mitotic neurons for normal memory formation function may occur through histone acetylation allowing more efficient transcription for LTM formation. The proteins JIL-1 and 14-3-3 are required for Elp3 binding to chromatin and the levels of histone H3K9 acetylation by Elp3 are significantly reduced in the absence of either protein (Karam et al. 2010). Interestingly, 14-3-3 proteins are also required for *Drosophila* memory formation (Philip et al. 2001; Skoulakis and Davis, 1996) and 14-3-3 proteins interact with Elp3 in nucleus during transcription elongation (Karam et al. 2010). However, genetic lesion of *Leonardo*, the gene encoding *Drosophila* 14-3-3ζ, produces deficits in short-term olfactory memory in contrast to Elongator complex disruption. Furthermore, our immunohistochemistry experiments show that Elp3 is most abundance in the cytoplasm (Fig. 3C), although we cannot rule out a presence and function in the nucleus.

Elp3 is known to localize to the cytoplasm of motor neurons in third instar larvae on the presynaptic side of neuromuscular junction (Miskiewicz et al. 2011). There, it acetylates the ELKS family member Bruchpilot, an integral structural component of presynaptic release sites (Miskiewicz et al. 2011). One could speculate that the Elongator complex may participate in a transcription and translation-dependent reorganization of presynaptic terminals that may occur during LTM formation. Elp3 also acetylates α-tubulin (Creppe et al. 2009; Solinger et al. 2010) and it is possible that this role underlies its participation in LTM. The only α-tubulin expressed in *Drosophila* neurons is α-tubulin 67 (Venkei et al. 2006), but overexpression of *UAS-α-tubulin 67* did not rescue the deficit conferred by *elp3* RNAi expression in the MBn. This could mean that α-tubulin is not a target of Elp3. Or, it may not be the sole target required for LTM. Moreover, the acetylation of α-tubulin by Elp3 was not altered in the FD cerebrum and in several *IKBKAP*/Elp1 down-regulated cell lines and in *Drosophila* neurons (Cheishvili et al. 2011; Miskiewicz et al. 2011). Thus, the downstream targets of Elongator complex in cytoplasm for LTM remain a mystery. Yet, Elongator complex might be participating in protein translation required for LTM. One main cellular function of Elongator complex in the cytoplasm is translational regulation of gene expression via specific modifications of uridines at the wobble base position of tRNAs (Karlsborn et al. 2014; Glatt et al. 2016). Elongator's specific ncm5/mcm5 tRNA modification reaction is emerging as a major enzymatic function that could explain many of the diverse phenotypic outcomes associated with mutations in Elongator complex genes. These include stem cell maintenance and early development (Yoo et al. 2016), neurodegenerative diseases (Simpson et al. 2009) and FD (Anderson et al. 2001; Slaugenhaupt et al. 2001). This complexity also makes clear the difficulty in elucidating downstream, cytoplasmic targets for Elongator complex.

Materials and Methods

Experimental design and statistical tests

The experiments described here for behavioral, immunohistochemical, and functional imaging experiments in general utilized a "within genotype" experimental design. The same genotype acted as its own control with the experimental arm being fed RU486 (+RU486) and the control arm being fed without RU486 (–RU486). Some Western blot and q-RT-PCR experiments utilized a "between genotype" experimental design, due to the need to express transgenes more broadly (CNS-wide) for subsequent biochemical experiments. Much of the data is presented as bar graphs showing the mean and standard error of the mean. Statistical tests with all relevant parameters are described in each figure legend. Data from behavioral, immunohistochemical, and imaging experiments were analyzed using nonparametric statistical tests. The Wilcoxon test was used for evaluating significance from zero. A Kruskal–Wallis H statistic was computed when comparing different groups, followed by pairwise comparisons using Mann–Whitney or Dunn's Multiple Comparison Test.

Transgenic animals and fly culture

Flies were cultured on standard medium at room temperature and transferred to a 25°C incubator for RU486 feeding. Flies carrying the *uas* transgenes *elp3*^{RNAi106128}, *elp3*^{RNAi19470}, *elp1*^{RNAi109402}, and *elp1*^{RNAi45369} were obtained from the Vienna *Drosophila* RNAi

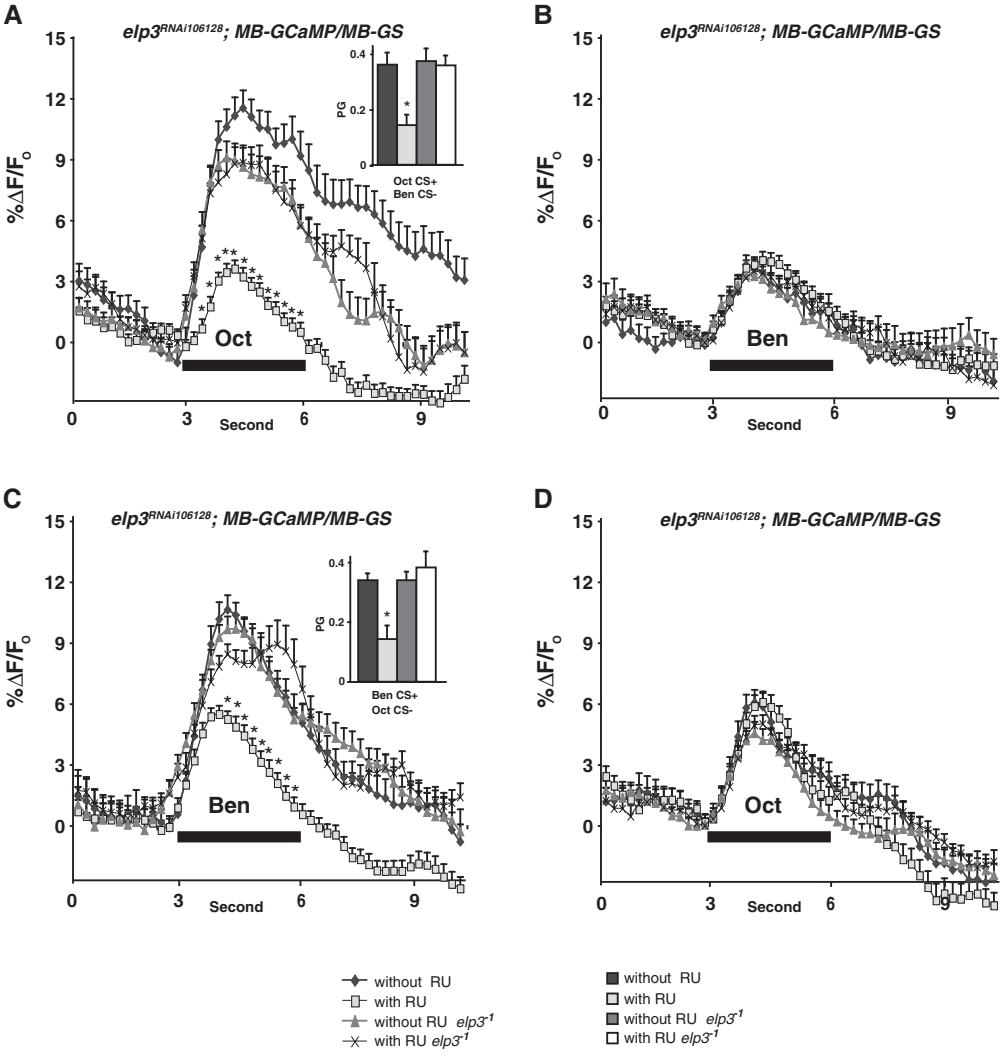

Figure 7. Expression of an *elp3* RNAi blocks the formation of a LTM trace. The block is rescued with expression of a wild-type *elp3* transgene. (Insert in *A*) Performance of flies expressing only *elp3*^{RNAi106128} (*uas-elp3*^{RNAi106128}/+; *MB-GS, uas-dcr2/MB-GCaMP*) *or* coexpressing *elp3*^{RNAi106128} and wild-type *elp3*$^{-1}$ in adult MBn (*uas- elp3*^{RNAi106128}/*uas-elp3*$^{-1}$*; MB-GS, uas-dcr2/MB-GCaMP*) after single-odor conditioning using OCT as CS+. Flies expressing only *elp3*^{RNAi106128} in the MBn exhibited a significant decrement in performance compared to flies of the same genotype that remained uninduced and other groups tested (Kruskal–Wallis multicomparison, $P \leq 0.0129$; Mann–Whitney pairwise comparisons $P < 0.0001$, $n = 6$ per group). Flies expressing both *elp3*^{RNAi106128} and the wild-type *elp3* transgene in the adult MBn exhibited performance levels that were indistinguishable from flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P = 0.4346$, $n = 6$ per group). A small fraction of the trained animals was removed prior to behavioral testing and used for functional imaging experiments shown in (*A*) and (*B*). (*A*) Calcium responses in the α branch of the α/β MBn across time during a 3 sec presentation of the CS+ (OCT) at 24 h after conditioning with OCT as the CS+. Flies expressing *elp3*^{RNAi106128} in the MBn exhibited an attenuated calcium response to the CS+ measured by $\%\Delta F/F_o$ compared to flies of the same genotype that remained uninduced and other groups tested at the same time (Kruskal–Wallis multiple-comparison, $P < 0.05$; Mann–Whitney pairwise comparisons, $P < 0.05$, $n = 7$–10). There was no significant difference in response to the CS+ (OCT) between flies expressing both *elp3*^{RNAi106128} and wild-type *elp3* and flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P \geq 0.4523$, $n = 7$–10). (*B*) Calcium responses in the α branch of the α/β MBn across time during the presentation of the CS– (BEN) at 24 h after conditioning with OCT as the CS+. There were no significant differences in response to the CS– (BEN) across the time of the 3-sec odor presentation between the four groups (Kruskal–Wallis multiple-comparison, $P \geq 0.6673$; Mann–Whitney pairwise comparisons, $P \geq 0.1943$, $n = 7$–10). (Insert in *C*) Performance of flies expressing only *elp3*^{RNAi106128} (*uas-elp3*^{RNAi106128}/+; *MB-GS, uas-dcr2/MB-GCaMP*) *or* coexpressing *elp3*^{RNAi106128} and wild-type *elp3*$^{-1}$ in adult MBn (*uas- elp3*^{RNAi106128}/*uas-elp3*$^{-1}$*; MB-GS, uas-dcr2/MB-GCaMP*) after single-odor conditioning using BEN as CS+. Flies expressing only *elp3*^{RNAi106128} in the MBn exhibited a significant decrement in performance compared to flies of the same genotype that remained uninduced and other groups tested (Kruskal–Wallis multicomparison, $P \leq 0.0129$; Mann–Whitney pairwise comparisons, $P < 0.0001$, $n = 6$ per group). Flies expressing both *elp3*^{RNAi106128} and the wild-type *elp3* transgene in the adult MBn exhibited performance levels that were indistinguishable from flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P = 0.4346$, $n = 6$ per group). A small fraction of the trained animals was removed prior to behavioral testing and used for functional imaging experiments shown in (*C*) and (*D*). (*C*) Calcium responses in the α branch of the α/β MBn across time during a 3-sec presentation of the CS+ (BEN) at 24 h after conditioning with BEN as the CS+. Flies expressing *elp3*^{RNAi106128} in the MBn exhibited an attenuated calcium response to the CS+ measured by $\%\Delta F/F_o$ compared to flies of the same genotype that remained uninduced and other groups tested at the same time (Kruskal–Wallis multiple-comparison, $P < 0.05$; Mann–Whitney pairwise comparisons, $P \leq 0.05$, $n = 7$–10). There was no significant difference in response to the CS+ (BEN) between flies expressing both *elp3*^{RNAi106128} and the wild-type *elp3* transgene and flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P \geq 0.1564$, $n = 7$–10). (*D*) Calcium responses in the α branch of the α/β MBn across time during the presentation of the CS– (OCT) at 24 h after conditioning with BEN as the CS+. There were no differences between the four groups (Kruskal–Wallis multiple-comparison, $P = 0.3246$; Mann–Whitney pairwise comparisons, $P \geq 0.1437$, $n = 7$–10). The prolonged calcium response after odor stimulation may result from a slow dissociation of calcium and GCaMP. The dip below zero on $\%\Delta F/F_0$ may result from bleaching.

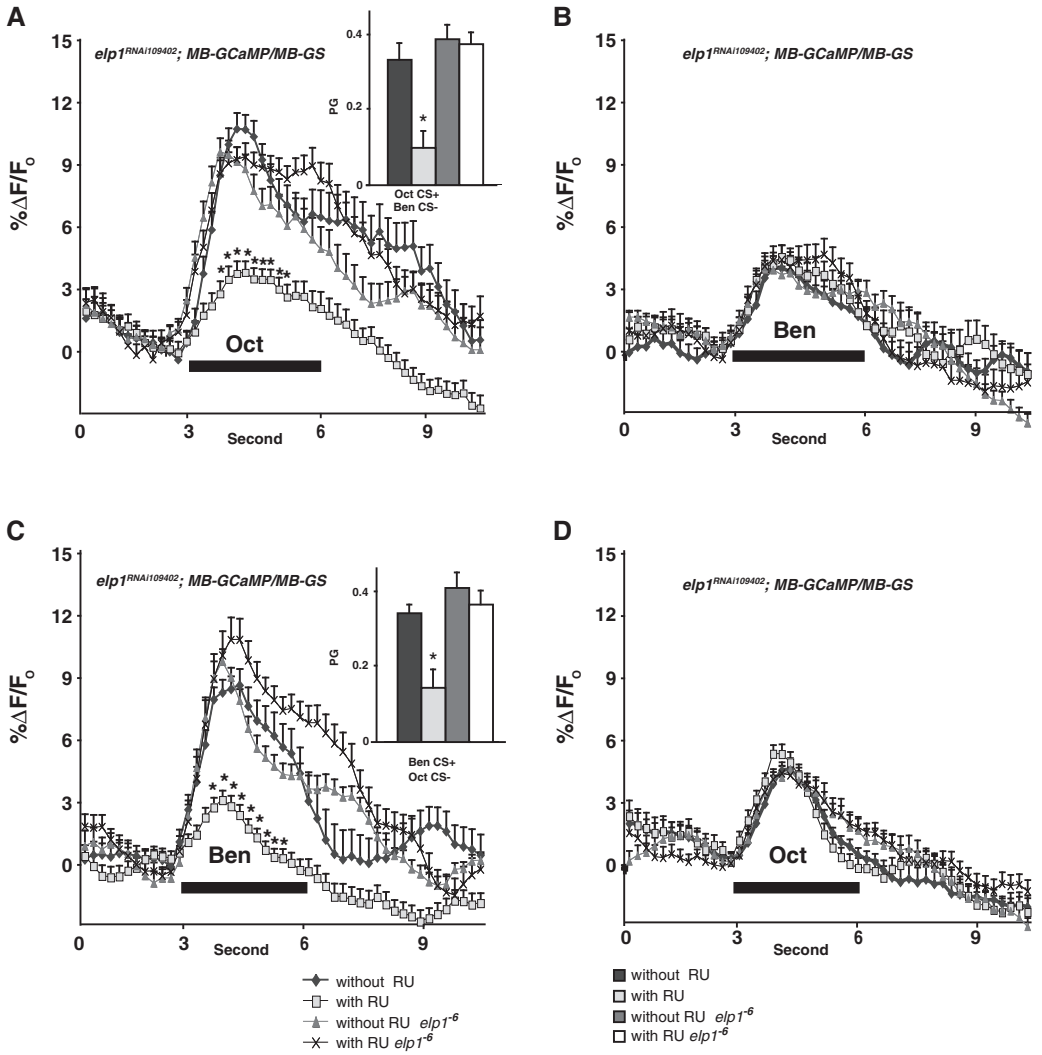

Figure 8. Expression of an *elp1* RNAi blocks the formation of a LTM trace. The block is rescued with expression of a wild-type *elp1* transgene. (Insert in *A*) Performance of flies expressing only *elp1*^{RNAi109402} (*uas-elp1*^{RNAi109402}/+; *MB-GCaMP/MB-GS, uas-dcr2*) *or* coexpressing *elp1*^{RNAi109402} and wild-type *elp1*$^{-6}$ in adult MBn (*uas- elp1*^{RNAi109402}/*uas-elp1*$^{-6}$*; MB-GCaMP/MB-GS, uas-dcr2*) after single-odor conditioning using OCT as CS+. Flies expressing only *elp1*^{RNAi109402} in the MBn exhibited a significant decrement in performance compared to flies of the same genotype that remained uninduced and other groups tested (Kruskal–Wallis multiple-comparison, $P \leq 0.0030$; Mann–Whitney pairwise comparisons, $P < 0.0001$, $n = 6$ per group). Flies expressing both *elp1*^{RNAi109402} and wild-type *elp1* in the adult MBn exhibited performance levels that were indistinguishable from flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P = 0.3364$, $n = 6$ per group). A small fraction of the behaviorally trained animals was removed prior to testing and used for functional imaging experiments shown in panels (*A*) and (*B*). (*A*) Calcium responses in the α branch of the α/β MBn across time during a 3 sec presentation of the CS+ (OCT) at 24 h after conditioning with OCT as the CS+. Flies expressing *elp1*^{RNAi109402} in the MBn exhibited an attenuated calcium response to the CS+ measured by $\%\Delta F/F_o$ compared to flies of the same genotype that remained uninduced and other groups tested at the same time (Kruskal–Wallis multiple-comparison, $P < 0.05$; Mann–Whitney pairwise comparisons, $P < 0.05$, $n = 7$–10). There was no significant difference in response to the CS+ (OCT) between flies expressing both *elp1*^{RNAi109402} and wild-type *elp1* and flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P \geq 0.1438$, $n = 7$–10). (*B*) Calcium responses in the α branch of the α/β MBn across time during the presentation of the CS– (BEN) at 24 h after conditioning with OCT as the CS+. There were no significant differences in response to the CS– (BEN) across the time of the 3 sec odor application among the four groups (Kruskal–Wallis multiple-comparison, $P = 0.5647$; Mann–Whitney pairwise comparisons, $P \geq 0.2877$, $n = 7$–10). (Insert in *C*) Performance of flies expressing only *elp1*^{RNAi109402} (*uas-elp1*^{RNAi109402}/+; *MB-GCaMP/MB-GS, uas-dcr2*) *or* coexpressing *elp1*^{RNAi109402} and wild-type *elp1*$^{-6}$ in adult MBn (*uas- elp1*^{RNAi109402}/*uas-elp1*$^{-6}$*; MB-GCaMP/MB-GS, uas-dcr2*) after single-odor conditioning using BEN as CS+. Flies expressing only *elp1*^{RNAi109402} in the MBn exhibited a significant decrement in performance compared to flies of the same genotype that remained uninduced and other groups tested (Kruskal–Wallis multiple-comparison, $P \leq 0.0030$; Mann–Whitney pairwise comparisons, $P < 0.0001$, $n = 6$ per group). Flies expressing both *elp1*^{RNAi109402} and wild-type *elp1* in the adult MBn exhibited performance levels that were indistinguishable from flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P = 0.3364$, $n = 6$ per group). A small fraction of the behaviorally trained animals was removed prior to testing and used for functional imaging experiments shown in panels (*C*) and (*D*). (*C*) Calcium responses in the α branch of the α/β MBn across time during a 3 sec presentation of the CS+ (BEN) at 24 h after conditioning with BEN as the CS+. Flies expressing *elp1*^{RNAi109402} in the MBn exhibited an attenuated calcium response to the CS+ measured by $\%\Delta F/F_o$ compared to flies of the same genotype that remained uninduced and other groups tested at the same time (Kruskal–Wallis multiple-comparison, $P < 0.5$; Mann–Whitney pairwise comparisons, $P < 0.05$, $n = 7$–10). There was no significant difference in response to the CS+ (BEN) between flies expressing both *elp1*^{RNAi109402} and wild-type *elp1* and flies of the same genotype that remained uninduced (Mann–Whitney pairwise comparisons, $P \geq 0.0678$, $n = 7$–10). (*D*) Calcium responses in the α branch of the α/β MBn across time during the presentation of the CS– (OCT) at 24 h after conditioning with BEN as the CS+. There were no significant differences in response to the CS– (OCT) across the time of the 3-sec odor application among the four groups (Kruskal–Wallis multiple-comparison, $P \geq 0.2612$; Mann–Whitney pairwise comparisons, $P \geq 0.1563$, $n = 7$–10).

Center (VDRC). All lines were out-crossed to *w(CS10)* for six generations to normalize the genetic background before being used in behavioral experiments. The fly line MB-GS containing *uas*-MB-Gene-Switch (MB-GS) on third chromosome was the *P {MB-Switch}12–1* line as previously reported (Mao et al. 2004). The MB-GS line was used as driver for expression of the *uas-RNAi, -elp3* or *-elp1* overexpression, and *-dcr2* transgenes. *Uas*-dicer2 on the third chromosome (from VDRC) was used to increase the potency of RNAi knockdown. The *elp3* and *elp1* cDNAs were cloned from *w(CS10)* flies using RT-PCR and confirmed by sequencing. Multiple independent *uas-elp3* and *uas-elp1* transgenic lines were generated by transforming *w(CS10)* flies with the *pUAST*-vector containing *elp3* or *elp1* cDNAs. Two independent *elp3* transgenic lines, *uas-elp3*$^{-1}$ and *uas-elp3*$^{-5}$, and two *elp1* lines, *uas-elp1*$^{-6}$ and *uas-elp1*$^{-5}$, were selected for subsequent use based on potency. The *MB-GCaMP* line was generated by transforming *w(CS10)* flies with a *pUAST* vector containing the 247-bp MBn enhancer from the *Dmef2* gene upstream of a GCaMP 1.3 cDNA. This line exhibited expression of GCaMP in the MBn like the MBn *Gal4* line p247 and was used for all imaging experiments.

Behavioral assays

Two-odor *Drosophila* olfactory memory was assayed using olfactory classical conditioning as described (Beck et al. 2000). Flies were exposed to two odors in succession, one odor (the CS+) paired with electric shock pulses (the US) followed by a second odor (the CS–) without electric shock. The flies were then presented to the two odors in a T-maze, and one-half of the performance index (PI) computed as the fraction of flies avoiding the CS+ minus the fraction avoiding the CS– divided by the total number of flies in both arms. Avoidance index (AI) was computed as the fraction of flies avoiding the odor minus the fraction avoiding the air divided by the total number of flies in both arms. The overall PI was the average of two half-PIs, with each half-PI obtained from using each of the two odors as the CS+ and the opposite odor as the CS–. Thus, the PI provides an index for the performance gains as averaged across using two different odors as the CS+.

We modified this protocol to obtain single-odor performance gains (PG) by substituting a naïve control for each trained group as previously reported (Yu et al. 2006). The "trained" group of flies was exposed to a CS+ and CS– odor as described above. The naïve group received the same handling and physical manipulations as the trained flies, including introducing them into a training tube, except they were not administered odor or electric shock while in the training tube. After training the "trained" group or mock training the naïve group, the flies were incubated at 25°C for the indicated times before testing against the CS+ and CS– in a T-maze. For each group of flies trained and tested with a specific CS+ odor, a naive group was tested simultaneously. The half-PI was then calculated for both the naive and trained group and the PI, or performance gain (PG), was obtained by subtracting the naive score from the score of the corresponding trained group. In all cases, only experiments where the naive flies exhibited naive performance scores that were not significantly different from zero (Wilcoxon test) were used. This assay allowed us to obtain an index of the performance gains due to conditioning with each specific odor as the CS+ so that these gains could be compared with the results obtained from functional imaging individual flies. Single-odor conditioning was used here only for 5× spaced training since imaging data was acquired only from these flies. All the genotypes assayed using this modified protocol were also trained using two-odor conditioning. We used 5× spaced training to generate LTM. Spaced training was performed with an interval of 15 min between each training cycle, whereas massed training was performed with no inter-trial interval. Spaced and massed training memory was tested 24 h after training. Three min or 3 h memory was tested at 3 min or 3 h after single cycle conditioning. All behavioral experiments presented utilized a "between group" design.

RU486 feeding

One-day old flies were collected and distributed between vials containing normal food and vials containing food supplemented with 200 μM RU486. Following a feeding period of 72 h at 25°C with daily transfers to fresh food vials with or without RU486, the flies were trained using single- or two-odor conditioning. After training, the flies were transferred to fresh food vials with or without RU486 and rested at 25°C for 3 h or 24 h before testing.

Molecular biology

Primers and TaqMan probes for quantitative PCR were designed and synthesized by Integrated DNA Technologies Assay-by-Design Service. The sequences of the primers and probe for each amplicon were as follows:

ACCGAATCAAAACAAGTGCTG (*elp3* forward primer); CTG AATGATCTCCCCGATGAC (*elp3* reverse primer); CGCCAGG CCCGACAGGTGCT (*elp3* probe);

AGCGGACAAGACACTTAAGG (*elp1* forward primer);

AGTAGATGCTGATTGCGAAGG (*elp1* reverse primer); TTGGGATGGTTACAGGTGCTCTC (*elp1* probe);

CACCAGTCGGATCGATATGCT (*rp49* forward primer);

ACGCACTCTGTTGTCGATACC (*rp49* reverse primer);

CATTTGTGCGACAGCTT (*rp49* probe).

Total RNA was isolated from fly heads using the TRIZOL reagent (Invitrogen) and reverse transcribed into cDNA using the SuperScript III first-strand synthesis system (Invitrogen). Four independent cDNA samples from different vials of each genotype were prepared from four independent samples of total RNA. For each independent cDNA sample, quantitative PCR was performed in duplicate to measure *elp1*, *elp3*, and/or *rp49* RNAs. The level of *elp3* or *elp1* transcript was first normalized to the loading control (*rp49*) and then to a *Gal4*^{c155}/+ control.

Antibody and Western blots

To generate a polyclonal antibody against Elp3, we amplified the *elp3* cDNA sequence corresponding to amino acid sequence 62–153 of the protein by PCR and subcloned this sequence inframe with GST protein coding sequences from the bacteria expression vector *pGEX-4T-1*. The resulting construct was sequenced and the fusion protein was subsequently expressed in *Escherichia coli* and purified using a GST fusion purification column (Thermo Scientific). The purified protein was used to raise anti-Elp3 antisera from rabbits (Open Biosystems). Elp3 polyclonal antibodies were purified from terminal bleed (day 96) antisera using HiTrap NHS activated Sepharose columns (GE Healthcare).

Fly heads were collected and homogenized over liquid nitrogen, centrifuged and the supernatant dissolved in Laemmli sample buffer with 5% β-mercaptoethanol (Bio-Rad Laboratories). The supernatant were boiled for 5 min and the supernatant equivalent to three fly heads was loaded onto each lane of a 4%–20% gradient precast SDS–PAGE gel (Bio-Rad Laboratories). After electrophoresis, the protein was transferred onto a PVDF membrane (Bio-Rad) and blotted with 1:200 anti-Elp3, 1:50,000 anti-CSP, 1:3000 anti-Nrg, 1:3000 anti-Elav antibodies and secondary antibodies (Abcam), and the signal detected using the Super signal west Pico Chemiluminescent detection kit (Pierce). The average grayscale intensity of the relevant protein band was measured with NIH Image J software. For each line, the *elp3/Elav* ratio was normalized to the wild-type (*wCS10* or *c155-Gal4*) sample.

Immunohistochemistry

Adult brains were dissected in freshly prepared phosphate buffered saline (PBS, pH 7.4), at the room temperature. They were then fixed in PBS containing 4% paraformaldehyde and 0.3% Triton X-100 for 1 h. They were washed with PBS containing 0.3% Triton X-100 for 6 × 10 min. Samples were incubated in blocking buffer (5% normal goat serum in PBS containing 0.3% Triton X-100) overnight at 4°C. They were incubated with primary antibody diluted in blocking buffer for 48 h at 4°C. After washing 6 × 10 min, the samples were incubated with secondary antibody diluted in blocking buffer overnight at 4°C. They were washed 6 × 10 min at 4°C and mounted in Vectashield (Vector Laboratories). The primary antibodies used include rabbit anti-elp3 (1:25) and mouse

anti-Dlg (1:100). Secondary antibodies were goat anti-rabbit IgG and goat anti-mouse IgG conjugated with Alexa Fluor 488, or 633 (Molecular Probes, all at 1:500). Images were collected using a 20× dry objective of a Leica TCS SP5 confocal microscope. The step size for z-stacks during imaging was generally 1 μm or less, with images collected typically at 1024 × 1024 pixel resolution.

Functional cellular imaging

We performed functional imaging according to previously described protocols (Yu et al. 2005, 2006). Flies containing both MB-GCaMP and *elp3* or *elp1* RNAi with or without an additional transgene of interest (*UAS-elp3* or *UAS-elp1*) were separated before behavioral testing from the remainder of the trained flies. The bulk of the trained flies were tested for behavioral memory as described above. Those removed for functional imaging were mounted in pipette tips and their exposed heads secured to the tip opening with silicon cement. To expose the brain, a small region of cuticle was removed from the top of the head capsule and the exposed area covered with a piece of plastic wrap. Confocal imaging was performed by mounting the flies under the 20× objective of a Leica TCS confocal microscope and imaged with a 488 nm excitation laser. The emitted light was collected from 505 to 535 nm. Two criteria were used to ensure that the same volume of the α MB lobe was imaged between flies. First, the complete mediolateral extent of the α lobe needed to be visible in the baseline image for functional imaging to continue. If not, the fly was discarded and another was prepared. Second, the bulbous tip of the α lobe was scanned in the z-plane to find the most intense focal plane, which occurred when the focus was centered on the midpoint of the α tip in the dorso-ventral axis. Odorants were spread on a small piece of filter paper inside a syringe barrel that was placed in line with pressurized air flowing at a rate of 100 mL/min. Concentrated odorants were diluted 10-fold in mineral oil. Odorant delivery was accomplished using a three-way Teflon valve under the control of a programmable timer, such that fresh air could be delivered to the animals for a determined period of time with an instantaneous switch to odor-laced air without altering the overall flow rate. Electric shock pulses were applied to the fly's abdomen. A total of 12 pulses of electric shock at 90 V was delivered with each shock lasting 1.25 sec. Conditioned flies were collected after training and tested at 24 h after training and tested for calcium influx into the MB axons when the CS+ and CS− odors were delivered at 5 min intervals.

Images were collected at approximately five frames per sec at a resolution of 256 × 256 pixels, followed by image data analysis as described previously (Yu et al. 2005, 2006). Regions of interest were circumscribed, and a pseudocolor image of the $\%\Delta F/F_0$ ratio was produced. The value F_o was calculated for each pixel within the region of interest as the fluorescence before odor application as averaged over five successive frames. The value ΔF was calculated for each pixel within the region of interest as the difference between the maximum average intensity during the 3 sec odor application for five successive frames and F_0.

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Author contributions: D.Y. and R.L.D. conceived and designed the experiments. D.Y. performed the behavioral and functional imaging experiments. Y.T. performed real time PCR experiments. M.C. performed the Western blotting, and M.C. and S.T. performed the immunohistochemistry. D.Y. and R.L.D. wrote the paper.

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