CREBA and CREBB in two identified neurons gate long-term memory formation in Drosophila

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Episodic events are frequently consolidated into labile memory but are not necessarily transferred to permanent long-term memory (LTM). Regulatory mechanisms leading to LTM formation are poorly understood, however, especially at the resolution of identified neurons. Here, we demonstrate enhanced LTM following aversive olfactory conditioning in Drosophila when the transcription factor cyclic AMP response element binding protein A (CREBA) is induced in just two dorsal-anterior-lateral (DAL) neurons. Our experiments show that this process is regulated by protein–gene interactions in DAL neurons: (1) crebA transcription is induced by training and repressed by crebB overexpression, (2) CREBA bidirectionally modulates LTM formation, (3) crebA overexpression enhances training-induced gene transcription, and (4) increasing membrane excitability enhances LTM formation and gene expression. These findings suggest that activity-dependent gene expression in DAL neurons during LTM formation is regulated by CREB proteins.

Long-lasting memory of the aversive olfactory task is uniquely produced after repetitive spaced training. Genetic and pharmacological dissections have established that this LTM is dependent on CREB-activated gene expression and the synthesis of new proteins (6, 8). Neural output from the MB is required for LTM formation (and retrieval), and here too several classes of extrinsic (mushroom body output [MBON]) neurons with projections to various other neuropils have been described (7). Although these effector neurons contribute to an extended network involved in LTM formation, de novo synthesis of proteins is required in only a few of them. More recently, an integrated molecular and circuit-based regulation of memory consolidation is being realized in the fly. Our group has identified several neurons in which protein synthesis is required for LTM consolidation. We found that MB output to two DAL neurons in the larger olfactory memory circuit induces protein synthesis– and CREB-dependent LTM. Subsequent feedback from DAL to MB neurons then appears necessary for LTM retrieval (8, 9).

In Drosophila, crebA and crebB genes encode CREB family proteins, CREBA and CREBB (the latter also known as dCREB2) (10, 11). crebB generates no fewer than nine distinct isoforms that function as transcriptional repressors and one reported activator of gene regulation or single neurons | CREBA | CREBB

memory consolidation | gene regulation | single neurons

**Significance**

Most animals record only labile memories of single events, whereas the formation of persistent long-term memories (LTMs) usually requires recurrent experiences. Our study distinguishes these different memory types through a deconvolution of molecular/biochemical processes within specific neurons of an identified memory circuit. A training-responsive gene activator, CREBA, engages paired DAL neurons in this circuit by promoting protein synthesis–dependent LTMs, which can otherwise be antagonized by CREBB repressor proteins. Increased CREBA expression or elevated membrane excitability enhances LTMs even after only one training cycle. These findings exemplify a circuit gating mechanism via cellular changes in specific single neurons to distinguish one-time experiences from multiple sessions of learning for storage as persistent memory.

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Activator and repressor crebB transgenes encoding these isoforms have been shown to enhance or impair LTM formation, respectively (13–16). In contrast, crebA encodes only one protein isoform, which is homologous to mammalian CREB3L1 and has been shown to function as a leucine-zipper transcription factor essential for embryonic development (10, 17). A role for CREBA in adult memory formation has not yet been shown.

Fig. 1. crebA expression in DAL neurons was enhanced after 10×S training and repressed by CREBB. (A) CREBA in dissected brains (green), counterstained with anti-DLG immunostaining (magenta). Arrows = DAL neurons. (Scale bar, 20 μm.) (B) Single optical slices of DAL neuron. crebA expression (green) colocalized with CREBA antibody (red) (Top) and with DDC antibody (red) (Middle) and G0431-Gal4 expression (green) colocalized with CREBA antibody (red) (Bottom). (Scale bar, 10 μm.) (C) crebA promoter activity after 1×, 3×M, and 3×S training with both MCH and OCT as reported by de novo KAEDE fluorescent protein synthesis (Left), estimated by the ratio of new (green, 488 nm) and preexisting (red, 561 nm) proteins (% ΔF/ ¯F0) (Right). For each brain, single DAL neuron optical slices were imaged under identical conditions. Training effects of 1× compared with 3×M (Top) and effects of 3×S compared with 3×M (Middle) were not significant. The crebA promoter was activated after 10×S but not 10×M (Bottom). (Scale bar, 10 μm.) (D) crebA activity was repressed by crebB overexpression (+CREBB) after 10×S as compared with 10×M training. (Scale bar, 10 μm.) Bars represent mean ± SE, n ≥ 8/bar. **P < 0.01; ***P < 0.001.
Here, we demonstrate a biochemical gating mechanism at the single neuron level within a memory circuit in which antagonistic regulatory events and changes in neural excitability lead to LTM formation. Our results show that LTM formation depends on transcriptional activation by CREBA in DAL neurons. Endogenous "reporter" genes, calcium/calmodulin-dependent protein kinase II (CaMKII) and period (per), are up-regulated specifically during recurrent learning (spaced training), by increased neuronal excitability during LTM consolidation, or by ectopic overexpression of crebA. Moreover, we show that CREBB can antagonize transcriptional activation by CREBA in DAL neurons, suggesting that interactions between CREBA and CREBB in DAL neurons together modulate LTM formation in flies.

Results

**CREBA in DAL Neurons Positively Regulates LTM Formation.** We first established a cellular function for CREBA. In a mouse F9 teratocarcinoma cell culture assay, significant PKA-dependent transcriptional activation of a CRE-luciferase transgene by CREBA was observed (SI Appendix, Fig. S1). Further, this up-regulation was antagonized by cotransfection of a CREBB repressor. To identify CREBA-positive neurons in the fly brain, we obtained and confirmed a Gal4 insertion in the crebA gene (BG00224; Berkeley Drosophila Genome Project [BDGP]) (SI Appendix, Table S1). crebA-Gal4 driving a UAS-mCD8::GFP green fluorescent protein transgene revealed a sparse neuronal pattern that included the DAL neurons (Fig. 1A). These observations were verified by (1) immunostaining with CREBA antibody (Fig. 1B, Top and SI Appendix, Fig. S2A), (2) colocalized crebA expression with Dopa Decarboxylase (DDC) immunostaining known to label DAL neurons (Fig. 1B, Middle), and (3) CREBA immunostaining with the DAL neuron-specific driver G0431-Gal4 (Fig. 1B, Bottom) (8). Strong and specific expression in DAL neurons further motivated our investigation of CREBA in the context of LTM formation.

We used a photoconvertible fluorescent KAEDE reporter transgene in DAL neurons to monitor the effect of training on crebA expression. After converting preexisting green KAEDE into red via ultraviolet (UV) irradiation prior to training, we measured the accumulation of newly synthesized green KAEDE driven by crebA-Gal4 after training (8). New expression of crebA was not detected after one training session (1×S) or three spaced training sessions (3×S) (Fig. 1C, Top two rows) but was elevated after 10 spaced training sessions (10×S) (Fig. 1C, Bottom row). In a manner analogous to the in vitro
results above, we overexpressed crebB in DAL neurons and observed repression of 10xS training-induced crebA expression (Fig. 1D). Thus, CREBB appears to antagonize the transcriptional activity of crebA in vivo in DAL neurons.

Using adult stage-specific transgenic RNA interference (RNAi) targeted by the Gal4/UAS system to knock down CREBA in DAL neurons under the temporal control of a temperature-sensitive tub-Gal80ts encoded protein (the inhibitory effect of GAL80ts

![Graphs showing regulation of CaMKII and per promoters by CREBA and CREBB](image)

**Fig. 3.** CREBA bidirectionally regulates CaMKII and per transcription in DAL neurons. (A) The CaMKII promoter was activated in DAL neurons 24 h after 10xS but not after 10xM, as reported by de novo KAEDE fluorescent protein synthesis (Left) (refer to Fig. 1 for details). Elevated de novo KAEDE synthesis was suppressed following RNAi down-regulation of CREBA (CREBA, Right). (B) The per promoter was similarly activated in DAL neurons 24 h after 10xS but not 10xM (Left). This activity was also abolished by RNAi down-regulation of CREBA (Right). (C and D) By contrast, levels of CaMKII (Left) and per (Right) gene activation were elevated by crebA overexpression (+CREBA) after 3xS and 1x but not after 3xM. Flies were raised at 18 °C and then transferred to 30 °C to remove GAL4 inhibition by GAL80ts for 3 d before training. Refer to SI Appendix, Figs. S3 and S4 for representative images. Bars represent mean ± SE, n ≥ 4/bar. *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. 4. Increased DAL neuron membrane excitability activates genes and enhances LTM. (A) NaChBac overexpression (+NaChBac) increased DAL neurons excitability and elevated 1-d memory after 3×S (Left), persisting for at least 4 d (Center Left). Memory was unaffected at 18 °C after 3×S (Center Right). Memory was similarly unaffected after 3×M (Right). (B) NaChBac overexpression also elevated 1-d memory after 1× (Left), persisting for at least 4 d (Center). Memory was unaffected at 18 °C after 1× (Right). (C) NaChBac overexpression in DAL neurons had no effect on 1-d memory after 10×S. (D and E) crebA (Top), CaMKII (Middle), and per (Bottom) activity in DAL neurons as reflected by de novo KAED synthesis (see Fig. 1) was elevated by NaChBac overexpression after 3×S and 1×. Refer to SI Appendix, Fig. S5 for representative images. Bars represent mean ± SE, n ≥ 6/bar. *P < 0.05; ***P < 0.001.
protein on GAL4 expression at 18 °C is inactivated at 30 °C, we found an impairment in LTM formation after 10xS training (Fig. 2A, Left). We observed no further reduction in 1-d memory after spaced training when flies were fed the protein synthesis inhibitor cycloheximide (CXM) (Fig. 2A, Middle Left), indicating that the CREBA knockdown specifically blocked LTM (16). Control flies kept at 18 °C after 10xS training or at 30 °C after 10xM training were unaffected (Fig. 2A, Middle Right and Right).

These results suggested that CREBA is a positive regulator of LTM formation. We tested this notion directly by overexpressing a creb4 transgene and then subjecting flies to suboptimal training (3xS) (13). Along with crebA, we also tested two other putative activator transgenes and a repressor transgene encoded by crebB (13–16). Of these four candidate transgenes, adult stage-specific overexpression targeted to DAL neurons enhanced LTM only for creb4 (Fig. 2B, Left and SI Appendix, Fig. S2B). This effect of creb4 persisted for at least 4 d (Fig. 2B, Middle Left), while control flies kept at 18 °C after spaced training or at 30 °C after massed training were unaffected (Fig. 2B, Middle Right and Right). creb4-enhanced LTM was also observed after 10xS training and somewhat after 10xS training (Fig. 2 C and D).

Induced transgenic expression of creb4 yielded immunopositive signals concentrated in the nuclei of DAL neurons, compared with sparse cytosolic signals in control flies (SI Appendix, Fig. S2C). Induced transgenic expression of creb4RNAi, on the other hand, decreased immunopositive signals in DAL neurons (but not in another central brain structure, the ellipsoid body [EB]) (SI Appendix, Fig. S2D). Together, these data suggest that LTM formation in DAL neurons is positively regulated by CREBA.

CREBA Modulates CaMKII and per Transcription. CaMKII and per are two genes, disruption of which are known to impair LTM and which appear to be transcriptionally regulated by CREBB (8). We were interested to know whether they were regulated by CREBA in DAL neurons during LTM formation. Consistent with their established roles in LTM formation, training-induced transcription of CaMKII-KAEDE or per-KAEDE in control flies was apparent after 10xS training. When transgenic creb4RNAi flies were subjected to 10xS training, however, transcription of these downstream genes was not observed (Fig. 3A and B and SI Appendix, Fig. S3A). Together, these data suggest that LTM formation in DAL neurons is positively regulated by CREBA.

Activity-Dependent Gene Expression in DAL Neurons Gates LTM Formation. In our experiments above, training appeared to induce the transcription of crebA and its downstream effector genes, CaMKII and per. Did training, then, produce these cellular responses via an increase in neuronal activity, as is the case in other model systems (18, 19)? We explored this notion by increasing membrane excitability (and thereby the propensity of neural activity) in DAL neurons via ectopic expression of a sodium channel transgene, UAS-NaChBac, under temporal control of tub-Gal80ts. When the

among intrinsic and extrinsic MB neurons. Persistent output from MBs then drives neural activity directly or indirectly between MB and DAL neurons via several MBONs. After CREBA-induced changes in gene expression within DAL neurons is complete, output from DAL neurons projecting back to the dendrites of pioneer axon-like neurons of the MB is required for memory retrieval.
inhibitory effect of GAL80Δ was inactivated at 30 °C before training, transgenic expression of NaChBac was sufficient to enhance 1-d memory after 5×S or 1× training (Fig. 4 A and B, Left). This manipulation had no effect on 1-d memory after 10×M training (Fig. 4 A, Right), after 10×S training (Fig. 4C) or after 3×S or 1× training in transgenic flies kept at 18 °C when active GAL80Δ blocked expression of the UAS-NaChBac transgene (Fig. 4A, Middle Right and Fig. 4 B, Right). Finally, enhanced memory after 3×S or 1× training persisted for at least 4 d, which is 3 d after flies were shifted back to 18 °C when GAL80Δ again blocked further expression of UAS-NaChBac (Fig. 4 A, Middle Left and Fig. 4 B, Middle). These latter results imply that training-induced neuronal activity is required to enhance LTM during acquisition and/or memory consolidation but not during memory retrieval. Finally, we extended these observations to the cellular level by showing that crebA–KAEDE, CaMKII–KAEDE, or per–KAEDE each was induced after 3×S or 1× training when NaChBac was overexpressed (Fig. 4 D and E and SI Appendix, Fig. S5 A and B). Thus, these transcriptional responses appear to be activity-dependent during LTM formation.

Discussion

CREBA and CREBB both are expressed in DAL neurons (20), and transgenic manipulations of CREBB have shown an impairment of 1-d memory after 10×S training (8). These studies, however, did not investigate a role for functional CREBA in DAL neurons and, in particular, did not query whether LTM might be enhanced. Here, we have focused on a role for CREBA in LTM formation. We first established in vitro that CREBA induced expression of a CRE-luciferase reporter gene in a PKA-dependent manner and was blocked by CREBB (SI Appendix, Fig. S1). Then, we used CREBA antibody, a DAL specific Gal4 driver and a crebA-driven KAEDE reporter to confirm that CREBA not only was expressed in DAL neurons but also responded transcriptionally to 10×S (but not 3×S or 1×) training (Fig. 1 A–C). In this in vivo context, we also showed that 10×S training-induced expression of crebA in DAL neurons was antagonized by overexpression of a crebB (repressor) transgene (Fig. 1D).

These observations suggested that CREBA in DAL neurons might serve as a positive regulator of protein synthesis-dependent LTM. Indeed, inducible transgenic manipulations of crebA only in DAL neurons were sufficient to impair 1-d memory after 10×S training (similar to inhibition of protein synthesis) using crebA RNAi or to enhance 1-d memory after 1× or 3×S training by overexpressing wild-type crebA. Importantly, LTM remained enhanced 4 d after 1× or 3×S training even when induction of transgenic crebA ceased 3 d earlier (Fig. 2). Together, these results suggest that CREBA in DAL neurons is involved in learning and/or memory consolidation but not necessarily in memory retrieval. CaMKII and per are two “downstream” genes that are CREB responsive, are expressed in DAL neurons and impair LTM when disrupted (8). Using CaMKII and per-driven KAEDE reporter transgenes, we have shown that expression of both genes is induced normally after 10×S training, is blocked after such training by induction of a crebA RNAi and is enhanced after 1× or 3×S training when a crebA transgene is inducibly expressed (Fig. 3). Here too, these transgenic manipulations are not required for CaMKII or per expression and LTM to persist for 4 d after training and 3 d after transgenic manipulations are blocked.

This role for CREBA in DAL neurons during learning and memory consolidation suggested that the transcriptional response might be activity dependent. We explored this possibility by expressing a NaChBac transgene in DAL neurons, which served to increase membrane excitability and presumably neuronal activity in response to training. We found that induced expression of NaChBac in DAL neurons was sufficient to enhance 1-d memory and to enhance expression of crebA, CaMKII, and per after 1× or 3×S training (Fig. 4). Together, these observations have suggested a model, which we describe in Fig. 5. The model illustrates how CREBA and CREBB interact to regulate transcription in DAL neurons and the activity-dependent transcriptional response to gate LTM formation.

CREB-dependent long-term memory formation first was shown in Drosophila using inducible transgenes, which were expressed throughout the fly (16). Acute expression of a transgenic crebB repressor blocked LTM after 10×S training, whereas similar manipulations of a synthetic crebB activator transgene enhanced LTM (13–16). An early attempt to identify specific neurons underlying LTM implicated MBs, wherein MB-specific transgenic expression of a crebb repres sor was reported to impair LTM after 10×S training (21). A subsequent study revealed, however, that this behavioral impairment derived from developmental defects in MB structure due to chronic expression of the crebb transgene. In contrast, induced expression of a crebb transgene only in adult-stage MBs did not impair LTM and did not produce any developmental defects (8). In neither study was a positive (CREB) regulator identified nor was enhanced LTM evaluated.

One-trial learning is usually insufficient to produce protein synthesis-dependent LTM, except for those experiences important for survival (22, 23). Here, we have demonstrated that LTM can form after a single training session when “memory genes” in DAL neurons are genetically manipulated. Learning-related and CREB-dependent changes in membrane excitability are well known and explain aspects of neuronal plasticity underlying memory consolidation (19). Regulation of ion channel genes by CREBA and CREBB transcription factors, for example, modulate plasticity in alcohol tolerance in Drosophila (24). CREB-dependent regulation of gene expression in DAL neurons appears sufficient to promote systems memory consolidation by modulating neural excitability. Further studies may elucidate whether neural circuits involved in motivation and attention also modulate DAL neurons during LTM formation (25, 26) and whether such prolonged neural activity also produces synaptic plasticity in DAL neurons (19, 27).

Materials and Methods

In this report we used an automated olfactory aversive learning task (8) and assessed LTM after elevating or blocking CREB-dependent gene activation in temporally and spatially targeted domains. Similarly, we bidirectionally modulated the expression of CREBA in DAL neurons to examine the effects on training-responsive gene transcription using a Gal4-targeted UV-sensitive KAED E reporter system (8). Spatial and temporal regulation of Na+ channel activity with transgene overexpression in DAL neurons was used to evaluate impacts of elevated membrane excitability on LTM and on training-responsive genes using the KAED E reporter system (8). See SI Appendix, Materials and Methods for details of fly strains, reagents, and all procedures.

Data Availability. All study data are included in the article and/or SI Appendix.

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