Published in final edited form as:

Neurobiol Learn Mem. 2015 February ; 0: 80–88. doi:10.1016/j.nlm.2014.11.010.

Spatio-Temporal *in vivo* Recording of dCREB2 Dynamics in *Drosophila* Long-Term Memory Processing

Jiabin Zhang^{a,b,#}, Anne K. Tanenhaus^{a,b,#}, John C. Davis^c, Bret M. Hanlon^c, and Jerry C. P. Yin^{b,d,*}

Jiabin Zhang: jzhang84@wisc.edu; Anne K. Tanenhaus: tanenhaus@wisc.edu; John C. Davis: jcdavis5@wisc.edu; Bret M. Hanlon: hanlon@stat.wisc.edu; Jerry C. P. Yin: jcyin@wisc.edu

^aNeuroscience Training Program, 1300 University Ave., University of Wisconsin-Madison, Madison, WI 53706

^bDepartment of Genetics, 3434 Genetics/Biotechnology, 425 Henry Mall, University of Wisconsin-Madison, Madison, WI 53706

^cDepartment of Statistics, University of Wisconsin-Madison, Madison, WI 53706

^dDepartment of Neurology, 1685 Highland Ave., University of Wisconsin-Madison, Madison, WI 53706

Abstract

CREB (cAMP response element-binding protein) is an evolutionarily conserved transcription factor, playing key roles in synaptic plasticity, intrinsic excitability and long-term memory (LTM) formation. The *Drosophila* homologue of mammalian CREB, dCREB2, is also important for LTM. However, the spatio-temporal nature of dCREB2 activity during memory consolidation is poorly understood. Using an *in vivo* reporter system, we examined dCREB2 activity continuously in specific brain regions during LTM processing. Two brain regions that have been shown to be important for *Drosophila* LTM are the ellipsoid body (EB) and the mushroom body (MB). We found that dCREB2 reporter activity is persistently elevated in EB R2/R4m neurons, but not neighboring R3/R4d neurons, following LTM-inducing training. In multiple subsets of MB neurons, dCREB2 reporter activity is suppressed immediately following LTM-specific training, and elevated during late windows. In addition, we observed heterogeneous responses across different subsets of neurons in MB $\alpha\beta$ lobe during LTM processing. All of these changes suggest that dCREB2 functions in both the EB and MB for LTM formation, and that this activity contributes to the process of systems consolidation.

© 2014 Elsevier Inc. All rights reserved.

*Corresponding Author: Jerry C.P. Yin, 3434 Genetics/Biotechnology, 425 Henry Mall, Madison, WI 53706, jcyin@wisc.edu, 608 262-5014.

#These authors contributed equally to this work.

The authors declare no competing financial interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

cAMP response element-binding protein; long-term memory; ellipsoid body; mushroom body; spatio-temporal recording; *in vivo* transcriptional activity

1. Introduction

Drosophila are able to form Pavlovian associations (Tully and Quinn, 1985), and the mechanisms of aversive olfactory learning and memory formation have been intensely studied (Keene and Waddell, 2007; Margulies et al., 2005; McGuire et al., 2005). After a single conditioning trial where an electric shock (unconditioned stimulus, or US) is coupled with a particular odor (conditioned stimulus, or CS), *Drosophila* can form a memory of the association between these two stimuli (Tully and Quinn, 1985). The newly formed memory will disappear within a day due to passive decay and/or interference (Shuai et al., 2010; Tully et al., 1994). However, flies trained with multiple spaced conditioning trials can form long-term memory (LTM) that lasts up to 7 days (Tully et al., 1994). *Drosophila* LTM formation requires activity of the transcription factor cAMP responsive-element binding protein (CREB or dCREB2 in flies) (Yin et al., 1994), which plays a critical, evolutionarily conserved role in the conversion of short-term memory (STM) to LTM (Alberini, 2009; Benito and Barco, 2010). Historically, CREB has been considered to be required around the time of training (Alberini, 2009; Yin et al., 1994). However, several studies in both mammals and *Aplysia* also suggest that the LTM-inducing training can produce biphasic effects on CREB activity, lasting for many hours (Bernabeu et al., 1997; Liu et al., 2011; Stanciu et al., 2001). Despite these indications, the inability to measure long-term changes in CREB activity over the course of memory processing has limited our understanding of CREB's role in LTM formation. Furthermore, in *Drosophila*, effects of genetic manipulations on LTM are generally tested behaviorally 24h after training. As a result, our understanding of the molecular events in memory formation is largely limited to this time window. In this study, we begin to address these issues through a continuous examination of dCREB2 activity for days following training that results in LTM formation.

Animal studies have shown that LTM usually involves multiple brain regions, and that different brain regions are recruited over time (Wang et al., 2006). In *Drosophila*, mushroom body (MB), ellipsoid body (EB) and dorsal anterior lateral (DAL) neurons have been shown to be important for LTM (Chen et al., 2012; Pascual and Preat, 2001; Wu et al., 2007), and a model of systems consolidation has been proposed (Dubnau and Chiang, 2013). Although the importance of dCREB2 in *Drosophila* LTM is clear, very little is known about the anatomical regions where dCREB2 activity is required to support LTM. In particular, two recent studies have raised the debate about the necessity of dCREB2 in the MB (Chen et al., 2012; Hirano et al., 2013). Measuring the real-time profile of dCREB2 activity in defined *Drosophila* brain tissues following LTM-specific training will provide significant insights into these issues. dCREB2 acts by binding cAMP-response elements (CRE) to modulate transcription. Previously, we generated a transgenic reporter consisting of a luciferase open reading frame under the control of multiple CRE elements (CRE-luc) that measures dCREB2-dependent gene expression *in vivo* (Belvin et al., 1999). We have recently

developed a FLP recombinase-activated version of this reporter (CRE-F-luc). To constrain the measurement of CREB activity to specific neuronal subsets, the Gal4-UAS and FLP/FRT systems are combined to spatially target reporter expression (Tanenhaus et al., 2012). Using this tool, we can continuously track dCREB2-responsive activity *in vivo* in brain regions of interest during the processing of LTM.

In this study, we examine when and where dCREB2 activity is altered in response to training that produces LTM in freely moving animals. We chose to focus on two prominent neuropils that are important for LTM, the EB and the MB. Our data shows a specific, long-lasting increase in dCREB2 reporter activity in the EB R2/R4m neurons during the consolidation of LTM. We also uncover bidirectional changes in dCREB2 reporter activity in different subsets of MB neurons. Combined with previous studies, we propose that dCREB2 activity is dynamically regulated across different anatomical regions, and that this might be involved in the systems consolidation of LTM.

2. Materials and Methods

2.1 *Drosophila* stocks

Gal4 lines OK107, 1471, c772, 17d and c320 were kindly provided by Dr. Yi Zhong (Cold Spring Harbor Laboratory). c42, c547 and c232 were generously shared by Dr. Vivek Jayaraman (Janelia Farm Research Campus).

2.2 Olfactory aversive conditioning

Aversive Pavlovian olfactory behavioral training and testing were performed as previously described (Drier et al., 2002). For 1x forward (1x FW) training, a group of ~100 flies was sequentially exposed to one odor (CS; 3-octanol or 4-methyl-cyclohexanol) paired with electric shock (US), and then the other odor without electric shock. 1x backward (1x BW) training is similar to 1x FW, but the US and CS are uncoupled, and the flies experience the US prior to CS. To generate LTM, flies were trained with 10x spaced forward (10x SFW) training trials (15 min interval between each training session). Flies trained with 10x spaced backward (10x SBW) training trials were used as control. To minimize any possible association between the US and CS in backward training trials, the time delay between these two stimuli was randomized for each training trial, i.e., 0s, 35s, 25s, 15s, 45s, 90s, 60s, 15s, 30s. To generate ARM (anesthesia resistant memory), flies were trained with 10x massed forward (10x MFW) training trials (no interval between each training session) and 10x massed backward (10x MBW) training was used as control. For LTM induction, 10x spaced training (lasting 3h) was always started at Zeitgeber Time=16 to generate robust LTM formation (Fropf et al., 2014). For other training protocols (i.e., 7x spaced training, 1x training and 10x massed training) that require less time, the onset of training was altered accordingly to ensure all the different trainings were completed at the same time of day (Zeitgeber Time=19).

2.3 *In vivo* luciferase assay

In vivo luciferase assays were performed as previously described (Tanenhaus et al., 2012). For each experiment, flies carrying a GAL4 driver line were crossed to UAS-FLP;CRE-F-

luc flies to generate the triply transgenic reporter lines used in each experiment. Immediately following training, flies were aspirated into individual wells of 96-well plates containing 25mM luciferin-fortified food. Plates were maintained under 12:12 light:dark conditions, and monitored hourly for bioluminescence using a TopCount microplate counter (PerkinElmer). To provide the best comparison across experiments, we limited our analysis to 96 hrs following training.

2.4 Statistical analysis

For statistical analysis of dCREB2 reporter data, we used a permutation test to test the null hypothesis that there was no effect of forward training (Ernst 2004, Good 2005). For this test, we needed to define windows of inspection. Because of the baseline circadian pattern of CREB activity, windows of inspection were set by day following training (day 0, 1, 2, 3, 4) and by daytime/nighttime interval. In most cases, each half-day interval contained 12 hourly measurements, except for the first and last bins. We use the following test statistic:

$$D = \sum_{i=1}^T \frac{|\bar{x}_i - \bar{y}_i|}{\bar{x}_i}$$

where $\bar{x}_i$ is the mean of the control (backward) group in bin i , $\bar{y}_i$ is the mean of the treatment (forward) group in bin i , and T is the total number of bins. The inclusion of the denominator accounts for baseline changes due to luciferin degradation and circadian oscillations.

For memory behavior, data were subjected to t-test (***: $p < 0.001$). Data were reported as mean $\pm$ SEM.

3. Results

3.1 Experimental design for behavioral training and *in vivo* luciferase assays

We previously generated a luciferase-based reporter system that allows us to record CRE-mediated transcriptional activity in specific brain tissues of freely moving *Drosophila* (Tanenhaus et al., 2012). In the present study, we use this tool to examine how LTM-specific training affects reporter activity. To ensure that the changes in reporter activity are due to LTM processing rather than a result of task-relevant stimuli (odor exposure and electric shock), we used backward training as a critical control. 1x FW training trial produces robust immediate memory, but 1x BW training does not (Fig. 1A, upper panel). To generate LTM, we trained flies with 10x spaced forward (10x SFW) training and used 10x spaced backward (10x SBW) training as a control condition (Fig. 1A, middle panel). The experimental design is summarized in Fig. 1B. Flies carrying both UAS-Flippase (UAS-FLP) and CRE-FRT-luciferase (CRE-F-luc) were crossed to different anatomically-specific GAL4 drivers. For each experiment, a group of flies expressing the bioluminescent reporter in neurons of interest (GAL4/UAS-FLP/CRE-F-luc) was randomly divided into two groups. The two groups of flies received 10x SFW training and 10x SBW training, respectively, and subsequent luciferase activity was measured *in vivo* as a readout of transcriptional activity. Since the only difference between the forward and backward training procedures is the shock-odor association, differences in activity between these two groups should reflect

associative LTM processing. The 10x SFW training protocol generates two types of consolidated memory, dCREB2-dependent LTM and dCREB2-independent ARM. To confirm that the observed changes following 10x SFW training are specific to dCREB2-dependent LTM, in some circumstances, we also trained groups of flies with 10x MFW and 10x MBW training protocol (Fig. 1A, lower panel), and then measured reporter activity. If similar changes were not observed after 10x massed training, it would demonstrate that the changes following 10x spaced training are specific to LTM. In this work, we examine two prominent brain regions that are important for LTM, the EB and the MB.

3.2 Reporter activity is increased in EB R2/R4m neurons for several days during LTM processing

Initially we focused on the EB, a brain region within the central complex that is composed of classes of ring neurons (R1, R2, R3, R4) (Renn et al., 1999). A previous study reported the requirement of NMDA receptors (NMDA-Rs) in EB neurons for LTM consolidation (Wu et al., 2007). However, the underlying molecular mechanisms are unknown. To test whether CRE-F-luc reporter activity in the EB is altered following LTM-inducing training, we expressed the reporter using different EB-GAL4 drivers, and then exposed the flies to either 10x SFW or 10x SBW training. When the c547-GAL4 driver was used to express the reporter in the R2 and medial R4 (R2/R4m EB) neurons (Renn et al., 1999), the level of CRE-F-luc activity is increased overall in 10x SFW-trained flies, throughout post-training day 1 and the daytime periods of day 2, and again increased during the daytime period of day 3 (Fig. 2A). To further confirm that the increases in CRE-F-luc activity are due to memory formation, we asked whether this effect is sensitive to training intensity. Training intensity affects the duration of olfactory LTM formation (Dudai, 1977). If the increase in reporter activity is indicative of LTM, it should be a function of the number of training trials. Therefore, we trained groups of flies with either 7x spaced training, which should produce weaker LTM formation, or a single training trial, which should not produce LTM. Indeed, compared to 10x SFW training, 7x SFW training produced a shorter increase in reporter activity, with a trend towards increased reporter activity lasting through the daytime period of day 1 (compare Fig. 2A with 2B). We found no significant differences when flies were trained with a single training trial (Fig. 2C). This correlation between training strength and reporter persistence further supports the link between changes in reporter activity and memory formation. The 10x SFW training protocol we use also produces a second form of consolidated memory, ARM, which is generally believed to be dCREB2-independent. In order to confirm that the elevation of reporter activity is related to LTM, rather than ARM, we examined the response following 10x massed training, which only produces ARM. We observed only very subtle changes in reporter activity following 10x massed training, which were statistically significant during the day 3 and day 4 daytime periods. This confirms that the persistent and dramatic increase in CRE-F-luc activity following 10x spaced training is specific to dCREB2-dependent LTM (Fig. 2D).

Are the changes in reporter activity anatomically specific? In the absence of a GAL4 driver, a small amount of reporter signal is detectable in UAS-FLP/CRE-F-luc flies, representing low levels of leaky expression. When these flies are exposed to the two training regimens, there is no significant difference between them, suggesting that the elevation of reporter

activity after training is not likely to be a general feature across the entire fly (Fig. 2E). This result suggests that our observed differences originate from certain tissues. When the reporter is expressed in a subset of photoreceptor cells (R1-6), which are not thought to contribute to olfactory memory, there were no detectable differences between the memory group and the control group (Fig. 2F). To examine the anatomical specificity within the EB, we made use of the c232-GAL4 line, which labels the R3 and distal R4 neurons (R3/R4d). These neurons are adjacent to the R2/R4m neurons (Renn et al., 1999), but have not been associated with olfactory LTM. In the EB R3/R4d neurons, 10x SFW training did not lead to a long-lasting increase in reporter activity (Fig. 2D). These data indicate that the LTM-associated persistent elevation of reporter activity in the EB is restricted to R2/R4m neurons. A second R2/R4m-specific driver line (c42) that was previously used to show the requirement for NMDA-Rs in LTM consolidation (Wu et al., 2007), produced effects that were similar to those seen with c547, with an overall increase throughout the first two post-training days that reached significance in the day 1 nighttime, and day 2 daytime and nighttime bins (Fig. 2H). Taken together, we have identified a long-lasting increase in reporter activity during LTM formation that is forward pairing dependent, modulated by training intensity, rest interval dependent, and anatomically specific. We therefore conclude that the long-lasting elevation in reporter activity in R2/R4m neurons is a property of LTM processing.

3.3 Reporter activity in MB neurons is dynamic during LTM processing

The MB is a central brain structure widely understood to be a key brain region for olfactory learning and different phases of memory formation, including LTM. MB neurons can be classified into three subtypes based on the distinctive axonal projections that form the resulting lobes: the α' β' , $\alpha\beta$, and γ MB neurons (Crittenden et al., 1998). ok107-GAL4 labels most of the MB neurons across the different lobes (Aso et al., 2009). Given the well-established function of dCREB2 in LTM formation, and the known role of the MBs in this process, we expected that CRE-F-luc reporter activity would be elevated in the MB following training. Surprisingly, the initial reporter activity in ok107-labeled MB neurons from the forward-trained group (memory group) was significantly *lower* than the activity from the control, backward-trained group, lasting through the daytime period of post-training day 1 (Fig. 3A). After this time period, we observed no significant differences between the two groups. To examine whether this suppression in reporter activity is specific to certain neuronal subsets, or across the whole MB, we surveyed multiple MB-Gal4 lines (for the expression pattern of the different MB drivers used in this study, see Table 1). c320-GAL4 expresses in $\alpha\beta$, α' β' and γ neurons, with strongest expression in the α' β' lobe. When the reporter was expressed under the control of c320-GAL4, the memory group showed little change in activity (Fig. 3B). However, the c739-GAL4 driver (which labels $\alpha\beta$ lobe neurons predominantly), and the 1471-GAL4 (which labels the γ lobe predominantly), both showed dramatic decreases in reporter activity immediately after the end of training. Using the c739-GAL4 driver, we observed a significant decrease in the first (day 1) daytime period. This returned to baseline during the nighttime period, though a trend towards decreased activity re-emerged during the subsequent (day 2) daytime period. Using the 1471-GAL4 driver, we observed a significant decrease during the initial (day 0) nighttime period, and day 1 daytime period, with this trend continuing into the day 1 nighttime period. Notably, the

relative suppression of reporter activity using c739 or 1471 is stronger than for ok107, though these drivers label fewer MB neurons (Fig. 3A, 3C, 3D). Intriguingly, c739-GAL4 reporter activity was increased in the forward group during the day-3 nighttime and day-4 daytime periods (Fig. 3C). Similarly, reporter activity under the control of 1471-GAL4 was significantly increased in the memory group during the daytime period of the third post-training day (Fig. 3D). These results suggest that LTM-inducing training results in distinct changes in dCREB2 activity over time, and across different MB neurons.

These heterogeneous changes in reporter activity led us to examine more specific GAL4 drivers. Because multiple studies have suggested that the $\alpha\beta$ lobe is important for LTM (Akmal et al., 2011; Huang et al., 2012; Pascual and Preat, 2001), we tested several GAL4 drivers that can drive gene expression in this lobe, including c747, c772 and 17d. The MB $\alpha\beta$ lobe can be further subdivided into the core, surface and posterior subdivisions (Aso et al., 2009), each of which may play distinct roles in memory processing (Huang et al., 2013; Huang et al., 2012; Pavlopoulos et al., 2008). Interestingly, in contrast to the early suppression that we observed with other drivers (Fig. 3A, C, D) we found that 10x SFW training resulted in an initial elevation in reporter activity in c747-GAL4-driven MB neurons, beginning immediately after training and lasting through the first 12h (Fig. 3E). A driver line that produces a similar pattern of expression (c772) showed a similar effect with a trend towards increased reporter activity in the forward group during the initial daytime period (Fig. 3F). Note that the fold-change in c772-expressing neurons is weaker (Fig. 3F), presumably because c772 also labels the γ lobe where reporter activity is suppressed (Fig. 3D and Table 1). The observed changes in CRE-F-luc reporter activity with c747/c772 drivers are very different from the pattern we observe for c739. To further verify that the decrease in c739 neurons and the increases in c747 and c772 neurons are LTM specific, we also trained the flies with 10 cycles of massed training. As expected, we found no significant differences between the memory and control groups when massed training was performed (Fig. 3G). To extend our characterization of the $\alpha\beta$ lobe, we utilized another GAL4 line, 17d, which has restricted but strong GAL4 expression in the core region of $\alpha\beta$ lobe. We observed no significant differences between groups in the 17d-labeled $\alpha\beta$ neurons following LTM-inducing training. Due to the lack of specific GAL4 lines, we were not able to precisely pinpoint the neurons in which the reporter activity is decreased. However, our data suggest that the responses in reporter activity are heterogeneous across different subsets of neurons in the $\alpha\beta$ lobe.

4. Discussion

In this study we take advantage of a conditionally-expressed CRE-luciferase reporter fly (Tanenhaus et al., 2012) to explore dCREB2 activity *in vivo* during olfactory LTM formation. While other bZIP transcription factors such as dCREB-A can bind CRE sequences (Smolik et al., 1992; Usui et al., 1993), CRE-luc reporter activity is essentially abolished in dCREB2 mutant (S162) flies (Belvin et al., 1999), and diurnal temporal dynamics in CRE-luc reporter activity are mirrored by temporal dynamics in dCREB2 protein isoforms (Fropf et al., 2014). Therefore, we believe the changes we observe in CRE-F-luc reporter activity are best interpreted as changes in dCREB2 activity. The CRE sequences we have used consist of symmetric sites (5'-TGACGTCA-3'), and dCREB2 binds

these sequences with very high affinity. The highest affinity sequences that dCREB-A binds to are unknown, but are likely to be variations of the symmetric sites. dCREB2 can also form heterodimers with other bZIP proteins (Fassler et al., 2002), and the preferred binding sites of the heterodimers is uncharacterized (but not likely to be the symmetric site). Therefore, we believe that a significant subpool (probably consisting of homodimers) of the total dCREB2 protein is responsible for the reporter activity that is measured.

A number of key observations strongly suggest that the changes in dCREB2 activity that we detect are LTM-specific. Behaviorally, the sustained changes in the EB R2/R4m neurons require forward pairing, their duration is a function of trial number, and the pattern we observe is specific to spaced training rather than massed training. These are properties of LTM formation that are shared amongst all animals, including flies. In addition, when the reporter is expressed without a driver or in anatomically irrelevant brain regions (e.g. photoreceptor cells, EB R3/R4d), there is no detectable difference in activity between forward and backward groups. These results establish a strong correlation between LTM-specific behavioral and anatomical parameters, and relative changes in reporter activity.

We detect an overall increase in dCREB2 activity during LTM processing in a small subset of EB neurons that surprisingly can persist for multiple days. The NMDA-Rs in these neurons are necessary for LTM consolidation (Wu et al., 2007), and our results support the possibility that these neurons (and dCREB2 activity within them) are involved in the consolidation/maintenance of late-LTM. CREB activity is usually associated with acute stimuli that result in adaptive changes, so it is surprising to detect changes in its activity that persist for long durations of time. What might these long-lasting changes reflect? Recent work in rodent models suggests that CREB controls intrinsic excitability, since acute increases or decreases in its activity produce parallel changes in excitability. This could partially be achieved through altering the expression of genes, e.g., ion channels (Dong et al., 2006). There are also reports demonstrating changes in neuronal excitability following memory formation in both invertebrate and vertebrate models (Liu et al., 2011; Oh et al., 2010; Sehgal et al., 2013). Of particular relevance are the observations that these changes can have significantly long durations, and that their persistence requires protein synthesis and the activity of the PKA (Oh et al., 2009) and MAPK (Cohen-Matsliah et al., 2007) signaling pathways. These requirements are reminiscent of the CREB signature for memory formation itself. Intriguingly, CREB expression has been reported to be elevated for many hours after the end of training in *Aplysia* (Liu et al., 2011), and this increase could contribute to the persistence of CREB activity in that model. Although we have not directly measured excitability following behavioral training, we believe this general view is most consistent with the existing literature on CREB's role in memory formation and neuronal function, and our data. This would suggest that excitability in the EB R2/R4m neurons is elevated for a number of days during the process of memory formation.

Our other major finding is the heterogeneity of dCREB2 activity amongst the different MB lobes and within the $\alpha\beta$ lobe itself, suggesting that dCREB2 may play distinct roles in different subdivisions of MB neurons. Broadly, we observe two types of responses following LTM training within the MB that depend on the specific GAL4 driver line. Using *c747* or *c772*, we observe an initial elevation in dCREB2 in the forward group. This

observation is consistent with the recruitment of dCREB2 activity to support memory formation within a subset of neurons in the MB. In contrast, with ok107, 1471, or c739 drivers, we observe an initial suppression in dCREB2 activity. We are confident that both the increases and decreases in dCREB2 activity that we detect are functions of LTM formation, since they are not seen when flies are trained with massed training. This heterogeneity that we observe in dCREB2 activity does not segregate simply along identifiable anatomical lines. Since our measurement is the net readout from the populations that our driver recruits, the use of lobe- or even sub-lobe-specific driver lines may show no effect when subpopulations are responding in opposite directions. Therefore, we were not able to precisely map the neurons in which dCREB2 activity is increased or decreased during LTM processing. This limitation is best illustrated when we consider the activity patterns of the $\alpha\beta$ drivers (c739, c747 and 17d). Table 1 describes their patterns of expression, and this summary is based on the number of neurons in each region that exhibit fluorescence when each driver is used to express an EGFP reporter (Aso et al., 2009). The core region of the $\alpha\beta$ lobes (17d) does not show much change in dCREB2 activity, indicating that the difference between c739 and c747 is likely to reside in the surface and/or posterior regions. It should also be noted that some MB drivers we used also have GAL4 expression in EB neurons (Aso et al., 2009). While we cannot definitively exclude contributions from outside the MB, to the best of our knowledge, these MB drivers do not drive gene expression in the R2/R4m subset of EB neurons. Therefore, we believe that dCREB2 activity here is not likely to contribute to the patterns we observe using MB drivers, and interpret these changes as representative of the complex nature of the MB itself.

The complex changes in the MB may in part explain the conflicting results of two recent studies that test the requirement for dCREB2 in the MB during memory formation (Chen et al., 2012; Hirano et al., 2013). While it has been suggested that gene dosage of an inhibiting transgene is responsible for the discrepant results (Hirano et al., 2013), another possibility is that pre-training induction or expression of an inhibiting transgene could facilitate or inhibit memory formation, depending upon the brain region. Since these experiments involved pan-MB drivers, subtle experimental parameters, such as the pre-training conditions could bias the results and result in blockade or not. Our results argue that general MB GAL4 lines are not specific enough for dissecting the spatial distribution of dCREB2 activity during memory formation. Further experimentation with more specific drivers should help to resolve these issues.

Our data support the notion of further functional subdivisions within MB lobes, and imply the complex and dynamic nature of dCREB2 functions in MB during LTM processing. Given the well-established positive roles of CREB in the formation of LTM, the suppressions that we observe in MB reporter activity are especially surprising. What could be the role for suppression of dCREB2 activity in LTM? Recent work in mammals has demonstrated that relative CREB activity, and relative neuronal excitability, can shape the allocation of memory encoding (Han et al., 2007; Yiu et al., 2014; Zhou et al., 2009). Therefore, one possibility is that suppression of dCREB2 activity decreases the excitability in certain MB neurons permitting new LTM formation. Presumably, this will prevent new stimuli that occur after the end of training from interfering with LTM processing. Notably, it has been suggested that the γ lobe is the gateway for US input to form olfactory conditioning

(Qin et al., 2012), and that the core region of $\alpha\beta$ lobe functions as a permissive gate for LTM formation (Huang et al., 2012). Interestingly, we also observed significant increases in dCREB2 activity using the 1471 and c739 drivers 3–4 days after the end of training, during the late phases of LTM, though further investigation is required to strengthen this observation and test its importance.

Another surprising observation is that post-training changes appear to be sensitive to time of day. dCREB2 is under circadian control across many brain regions (Tanenhaus et al., 2012; Fropf et al., 2014), which is reflected in baseline diurnal oscillations in dCREB2 activity that we observe in most of our experiments. However, the relative changes we observe in dCREB2 activity, or lack thereof, are sometimes themselves associated with particular daytime or nighttime periods. This could reflect a convergence of training-dependent regulation of dCREB2 activity with circadian-dependent coordination of dCREB2 activity.

We recently reported transient changes in dCREB2 protein localization following the end of spaced training (Fropf et al., 2013). Those data are most consistent with the rapid, acute role that dCREB2 (and all CREB proteins) play in adaptive changes. In this report we show post-training persistent changes in dCREB2 activity, differences in duration and directionality of activity, and possibly even changes in directionality over time within one set of neurons. We believe that dCREB2 activity is recruited acutely to mediate adaptive changes, and is also recruited chronically to sustain changes in neuronal state, such as excitability (Benito and Barco, 2010). Both of these types of mechanisms may be utilized over the course of systems processing.

Our current study suggests that dCREB2 activity is modulated in both EB and MB neurons during LTM processing. A recent study using the GRASP (GFP reconstitution across synaptic partners) system has reported bidirectional synaptic connections between the MB and the EB (Zhang et al., 2013). The MB is also synaptically connected with DAL neurons where dCREB2 is required for LTM formation (Chen et al., 2012). Taken collectively, all of these studies support a model of systems consolidation of *Drosophila* LTM (Dubnau and Chiang, 2013), and suggest that the neural network of *Drosophila* LTM might be much broader and more complex than previously thought. Our dCREB2 reporter system may be useful to explore new circuitry components and add them to this network. The involvement of multiple brain regions and the dynamic nature of dCREB2 activity within specific circuits lead to a great capacity for information processing. Given the complex nature of dCREB2 in LTM, new tools that can acutely manipulate dCREB2 activity in a precise spatial and temporal manner will be necessary to investigate its diverse functions.

Acknowledgments

This work was supported through NIH funding to JCPY (RO1s NS35575, NS063245). Additional funding support was provided through the Neuroscience Training Program. Hong Zhou provided important technical assistance.

References

- Akalal DB, Yu D, Davis RL. The long-term memory trace formed in the *Drosophila* alpha/beta mushroom body neurons is abolished in long-term memory mutants. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2011; 31:5643–5647. [PubMed: 21490205]

- Alberini CM. Transcription factors in long-term memory and synaptic plasticity. *Physiological reviews*. 2009; 89:121–145. [PubMed: 19126756]
- Aso Y, Grubel K, Busch S, Friedrich AB, Siwanowicz I, Tanimoto H. The mushroom body of adult *Drosophila* characterized by GAL4 drivers. *Journal of neurogenetics*. 2009; 23:156–172. [PubMed: 19140035]
- Belvin MP, Zhou H, Yin JC. The *Drosophila* dCREB2 gene affects the circadian clock. *Neuron*. 1999; 22:777–787. [PubMed: 10230797]
- Benito E, Barco A. CREB's control of intrinsic and synaptic plasticity: implications for CREB-dependent memory models. *Trends in neurosciences*. 2010; 33:230–240. [PubMed: 20223527]
- Bernabeu R, Bevilacqua L, Ardenghi P, Bromberg E, Schmitz P, Bianchin M, Izquierdo I, Medina JH. Involvement of hippocampal cAMP/cAMP-dependent protein kinase signaling pathways in a late memory consolidation phase of aversively motivated learning in rats. *Proceedings of the National Academy of Sciences of the United States of America*. 1997; 94:7041–7046. [PubMed: 9192688]
- Chen CC, Wu JK, Lin HW, Pai TP, Fu TF, Wu CL, Tully T, Chiang AS. Visualizing long-term memory formation in two neurons of the *Drosophila* brain. *Science*. 2012; 335:678–685. [PubMed: 22323813]
- Cohen-Matsliah SI, Brosh I, Rosenblum K, Barkai E. A novel role for extracellular signal-regulated kinase in maintaining long-term memory-relevant excitability changes. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2007; 27:12584–12589. [PubMed: 18003837]
- Crittenden JR, Skoulakis EM, Han KA, Kalderon D, Davis RL. Tripartite mushroom body architecture revealed by antigenic markers. *Learning & memory*. 1998; 5:38–51. [PubMed: 10454371]
- Dong Y, Green T, Saal D, Marie H, Neve R, Nestler EJ, Malenka RC. CREB modulates excitability of nucleus accumbens neurons. *Nature neuroscience*. 2006; 9:475–477.
- Drier EA, Tello MK, Cowan M, Wu P, Blace N, Sacktor TC, Yin JC. Memory enhancement and formation by atypical PKM activity in *Drosophila melanogaster*. *Nature neuroscience*. 2002; 5:316–324.
- Dubnau J, Chiang AS. Systems memory consolidation in *Drosophila*. *Current opinion in neurobiology*. 2013; 23:84–91. [PubMed: 23084099]
- Dudai Y. Properties of learning and memory in *Drosophila melanogaster*. *J Comp Physiol*. 1977; 114:69–89.
- Ernst MD. Permutation methods: A basis for exact inference. *Statistical Science*. 2004; 19:676–685.
- Fassler J, Landsman D, Acharya A, Moll JR, Bonovich M, Vinson C. B-zip proteins encoded by the *Drosophila* genome: evaluation of potential dimerization partners. *Genome Res*. 2002; 12:1190–1200. [PubMed: 12176927]
- Fropf R, Tubon TC Jr, Yin JC. Nuclear gating of a *Drosophila* dCREB2 activator is involved in memory formation. *Neurobiology of learning and memory*. 2013; 106:258–267. [PubMed: 24076014]
- Fropf R, Zhang J, Tanenhaus AK, Fropf WJ, Siefkes E, Yin JC. Time of day influences memory formation and dCREB2 proteins in *Drosophila*. *Frontiers in systems neuroscience*. 2014; 8:43. [PubMed: 24744705]
- Good, PI. Permutation, parametric and bootstrap tests of hypotheses. 3rd. Springer; 2005.
- Han JH, Kushner SA, Yiu AP, Cole CJ, Matynia A, Brown RA, Neve RL, Guzowski JF, Silva AJ, Josselyn SA. Neuronal competition and selection during memory formation. *Science*. 2007; 316:457–460. [PubMed: 17446403]
- Hirano Y, Masuda T, Naganos S, Matsuno M, Ueno K, Miyashita T, Horiuchi J, Saitoe M. Fasting launches CRTC to facilitate long-term memory formation in *Drosophila*. *Science*. 2013; 339:443–446. [PubMed: 23349290]
- Huang C, Wang P, Xie Z, Wang L, Zhong Y. The differential requirement of mushroom body alpha/beta subdivisions in long-term memory retrieval in *Drosophila*. *Protein & cell*. 2013; 4:512–519. [PubMed: 23722532]
- Huang C, Zheng X, Zhao H, Li M, Wang P, Xie Z, Wang L, Zhong Y. A permissive role of mushroom body alpha/beta core neurons in long-term memory consolidation in *Drosophila*. *Current biology : CB*. 2012; 22:1981–1989. [PubMed: 23063437]

- Keene AC, Waddell S. *Drosophila* olfactory memory: single genes to complex neural circuits. *Nature reviews Neuroscience*. 2007; 8:341–354.
- Liu RY, Cleary LJ, Byrne JH. The requirement for enhanced CREB1 expression in consolidation of long-term synaptic facilitation and long-term excitability in sensory neurons of *Aplysia*. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2011; 31:6871–6879. [PubMed: 21543617]
- Margulies C, Tully T, Dubnau J. Deconstructing memory in *Drosophila*. *Current biology : CB*. 2005; 15:R700–713. [PubMed: 16139203]
- McGuire SE, Deshazer M, Davis RL. Thirty years of olfactory learning and memory research in *Drosophila melanogaster*. *Progress in neurobiology*. 2005; 76:328–347. [PubMed: 16266778]
- Oh MM, McKay BM, Power JM, Disterhoft JF. Learning-related postburst afterhyperpolarization reduction in CA1 pyramidal neurons is mediated by protein kinase A. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:1620–1625. [PubMed: 19164584]
- Oh MM, Oliveira FA, Disterhoft JF. Learning and aging related changes in intrinsic neuronal excitability. *Frontiers in aging neuroscience*. 2010; 2:2. [PubMed: 20552042]
- Pascual A, Preat T. Localization of long-term memory within the *Drosophila* mushroom body. *Science*. 2001; 294:1115–1117. [PubMed: 11691997]
- Pavlopoulos E, Anezaki M, Skoulakis EM. Neuralized is expressed in the alpha/beta lobes of adult *Drosophila* mushroom bodies and facilitates olfactory long-term memory formation. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105:14674–14679. [PubMed: 18794519]
- Qin H, Cressy M, Li W, Coravos JS, Izzi SA, Dubnau J. Gamma neurons mediate dopaminergic input during aversive olfactory memory formation in *Drosophila*. *Current biology : CB*. 2012; 22:608–614. [PubMed: 22425153]
- Renn SC, Armstrong JD, Yang M, Wang Z, An X, Kaiser K, Taghert PH. Genetic analysis of the *Drosophila* ellipsoid body neuropil: organization and development of the central complex. *Journal of neurobiology*. 1999; 41:189–207. [PubMed: 10512977]
- Sehgal M, Song C, Ehlers VL, Moyer JR Jr. Learning to learn - intrinsic plasticity as a metaplasticity mechanism for memory formation. *Neurobiology of learning and memory*. 2013; 105:186–199. [PubMed: 23871744]
- Shuai Y, Lu B, Hu Y, Wang L, Sun K, Zhong Y. Forgetting is regulated through Rac activity in *Drosophila*. *Cell*. 2010; 140:579–589. [PubMed: 20178749]
- Smolik SM, Rose RE, Goodman RH. A cyclic AMP-responsive element-binding transcriptional activator in *Drosophila melanogaster*, dCREB-A, is a member of the leucine zipper family. *Mol cell biol*. 1992; 12:4123–4131. [PubMed: 1508208]
- Stanciu M, Radulovic J, Spiess J. Phosphorylated cAMP response element binding protein in the mouse brain after fear conditioning: relationship to Fos production. *Brain research Molecular brain research*. 2001; 94:15–24. [PubMed: 11597761]
- Tanenhause AK, Zhang J, Yin JC. In vivo circadian oscillation of dCREB2 and NF-kappaB activity in the *Drosophila* nervous system. *PloS one*. 2012; 7:e45130. [PubMed: 23077489]
- Tully T, Preat T, Boynton SC, Del Vecchio M. Genetic dissection of consolidated memory in *Drosophila*. *Cell*. 1994; 79:35–47. [PubMed: 7923375]
- Tully T, Quinn WG. Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *Journal of comparative physiology A, Sensory, neural, and behavioral physiology*. 1985; 157:263–277.
- Usui T, Smolik SM, Goodman RH. Isolation of *Drosophila* CREB-B: a novel CRE-binding protein. *DNA cell biol*. 1993; 12:589–595. [PubMed: 8397816]
- Wang H, Hu Y, Tsien JZ. Molecular and systems mechanisms of memory consolidation and storage. *Progress in neurobiology*. 2006; 79:123–135. [PubMed: 16891050]
- Wu CL, Xia S, Fu TF, Wang H, Chen YH, Leong D, Chiang AS, Tully T. Specific requirement of NMDA receptors for long-term memory consolidation in *Drosophila* ellipsoid body. *Nature neuroscience*. 2007; 10:1578–1586.

- Yin JC, Wallach JS, Del Vecchio M, Wilder EL, Zhou H, Quinn WG, Tully T. Induction of a dominant negative CREB transgene specifically blocks long-term memory in *Drosophila*. *Cell*. 1994; 79:49–58. [PubMed: 7923376]
- Yiu AP, Mercaldo V, Yan C, Richards B, Rashid AJ, Hsiang HL, Pressey J, Mahadevan V, Tran MM, Kushner SA, et al. Neurons are recruited to a memory trace based on relative neuronal excitability immediately before training. *Neuron*. 2014; 83:722–735. [PubMed: 25102562]
- Zhang Z, Li X, Guo J, Li Y, Guo A. Two clusters of GABAergic ellipsoid body neurons modulate olfactory labile memory in *Drosophila*. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2013; 33:5175–5181. [PubMed: 23516283]
- Zhou Y, Won J, Karlsson MG, Zhou M, Rogerson T, Balaji J, Neve R, Poirazi P, Silva AJ. CREB regulates excitability and the allocation of memory to subsets of neurons in the amygdala. *Nature neuroscience*. 2009; 12:1438–1443.

Highlights

1. We have analyzed a new reporter that allows us to track dCREB2 activity *in vivo*, in real time after training that produces memory formation.
2. In certain brain regions (the Ellipsoid Body), reporter activity increases immediately after training and stays elevated for days (relative to a control treatment).
3. The response of the reporter is varied and complex in the mushroom body, particularly in sub-regions of the $\alpha\beta$ lobes.
4. In parts of the $\alpha\beta$ lobes, reporter activity goes up immediately after training but returns to control values after about 12 hours.
5. In other parts of the $\alpha\beta$ lobes (and in the γ lobe), reporter activity goes down immediately after training and remains depressed for 1–2 days. After about 3–4 days, reporter activity in these regions increases relative to controls.
6. We interpret these changes to reflect changes in excitability after the end of training. The complex dynamics (both in terms of anatomy and timing) demonstrate parts of the process of systems consolidation.

Figure 1. Experimental design and behavioral training

(A) Training protocols used to generate immediate memory, LTM and ARM. For 1x FW, an odor (OCT or MCH) is paired with a train of shocks, alternating with an unpaired odor. For 1x BW training, the shock is presented before both odors. 1xFW generates robust immediate memory, while 1xBW does not (upper panel). 10x SFW and 10x SBW training contains 10 cycles of 1x FW and 1x BW, respectively, with a 15 minute rest interval between each cycle. 10x SFW training was used to generate LTM and 10x SBW was used as a critical control treatment (middle panel). 10x MFW training was used to generate ARM and 10x MBW was used as its control condition (lower panel). LTM and ARM were tested 24 hr after training. N=8 (approximately 200 flies for each N-of-1) for all the groups (** $p < 0.001$).

(B) The flow of the experimental design. A group of flies expressing the dCREB2 reporter in a specific brain region (GAL4/UAS-FLP/CRE-F-luc) was randomly divided into two groups. These two groups of flies were trained in parallel with 10x SFW and 10x SBW training, respectively, and bioluminescent reporter activity was measured beginning immediately following training. The differences in dCREB2 activity between these two groups were considered to be LTM related. In some circumstances, this LTM experiment was followed by an ARM (dCREB2-independent) experiment (10x MFW and 10x MBW) to rule out the involvement of ARM in these changes of dCREB2 activity.

Figure 2. dCREB2 activity in EB R2/R4m neurons following LTM-inducing training
(A) Long-lasting elevation of dCREB2 activity in EB R2/R4m neurons after 10x SFW training. *c547-GAL4* was used to drive the expression of dCREB2 reporter in EB R2/R4m neurons. Relative luciferase activity for *UAS-FLP/+;CRE-F-luc/c547-GAL4* flies is plotted over 4 days following 10x SFW (red), or 10x SBW training (blue). Daytime and nighttime periods are indicated below the graph using white and black bars, respectively. Means (points) and standard errors (ribbons) are plotted for each measurement. The line indicates a smoothed mean (average of 3 time points). In *c547*-labeled neurons (R2/R4m neurons), 10x

SFW training triggered a significant persistent increase in dCREB2 activity. (FW: n=13, BW: n=19).

(B) Following 7x SFW training, the increase of dCREB2 activity in EB R2/R4m neurons is weaker and shorter-lived than that following 10x SFW training. (FW: n=10, BW: n=12)

(C) 1x FW training does not produce any significant increases in dCREB2 activity following training. (FW: n=91, BW: n=91)

(D) 10x MFW does not lead to persistent increase of dCREB2 activity following training. Flies were trained with 10 cycles of 1x training without rest intervals between each training trial. (FW: n=96, BW: n=96)

(E) Increase of dCREB2 activity following LTM-inducing training is not a general response. Flies containing UAS-FLP and CRE-F-luc without GAL4 driver (UAS-FLP/+; CRE-F-luc/+) were subjected to 10x SFW or 10x SBW training. The detected luciferase activity represents the broad leaky expression of luciferase over the whole fly body. No statistically significant differences were observed between the two groups. (FW: n=48, BW: n=48)

(F) dCREB2 activity is not increased in the photoreceptor cells following LTM-inducing training. The expression of dCREB2 reporter is driven by ninaE (GMR) GAL4 driver (UAS-FLP/ninaE-GAL4; CRE-F-luc/+). (FW: n=95, BW: n=96)

(G) LTM-inducing training does not trigger increase of dCREB2 activity in R3/R4d neurons. dCREB2 reporter was expressed in EB R3/R4 neurons that are close to, but distinct from, R2/R4m neurons using c232-GAL4. No persistent increase of dCREB2 activity was seen in the memory group. (FW: n=40, BW: n=38).

(H) Persistent elevation of dCREB2 activity in c42-GAL4-driven R2/R4m neurons after 10x SFW training. (FW: n=25, BW: n=28)

Asterisks indicate statistical significance for each day/night bin ($p < 0.05$).

Figure 3. Spatio-temporal dynamics of dCREB2 activity in MB neurons following LTM-inducing training

(A) Suppression of dCREB2 activity in ok107-labeled MB neurons following LTM-inducing training. A pan-MB GAL4 driver was used to express dCREB2 reporter across most of the MB neurons. The ok107/UAS-FLP/CRE-F-luc flies were trained with 10x SFW or 10x SBW training. During the first day after training, the dCREB2 activity in the memory group was significantly lower compared to the control group. (FW: $n=65$, BW: $n=68$)

(B) No significant change in dCREB2 activity in c320-labeled (preferentially in α' β' lobe, but also in $\alpha\beta$ and γ lobe) MB neurons following LTM-inducing training. (FW: $n=93$, BW: $n=89$)

(C and D) In c739-labeled ($\alpha\beta$ lobe) and 1471-labeled (γ lobe) MB neurons, LTM-inducing training led to a dramatic early suppression and significant late elevation of dCREB2 activity. (C, FW: $n=48$, BW: $n=48$; D, FW: $n=61$, BW: $n=65$)

(E and F) Increase of dCREB2 activity in subdivisions of MB $\alpha\beta$ lobe labeled by c747 and c772, respectively, following LTM-inducing training. (E, FW: $n=44$, BW: $n=44$; F, FW: $n=41$, BW: $n=43$)

(G) 10x MFW training does not induce the changes in dCREB2 activity observed in c739/c747/c772-labeled $\alpha\beta$ neurons following 10x SFW training. Only the data of first day after training are shown. (c739, FW: $n=48$, BW: $n=48$; c747, FW: $n=47$, BW: $n=47$; c772 FW: $n=48$, BW: $n=48$).

(H) No significant changes in dCREB2 activity in 17d-labeled (preferentially in the $\alpha\beta$ lobe core subdivision) following LTM-inducing training. (FW: $n=44$, BW: $n=44$)

Asterisks indicate statistical significance for each day/night bin ($p < 0.05$).

Table 1

Expression pattern of MB GAL4 drivers used in Figure 3

MB GAL4	γ main	γ dorsal	$\alpha'\beta$ anterior	$\alpha'\beta'$ middle	$\alpha'\beta$ posterior	$\alpha\beta$ core	$\alpha\beta$ surface	$\alpha\beta$ posterior	EB
ok107	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	
c320		+++	+++++	++	+++++	++	++	+	+++
c739						+++++	+++++	+++++	++
1471	+++								+
c747	+++	+++	++	++	++	++	+++++	+++++	+
c772	+++++	+++++	++	++	++	+++++	+++++	+++++	++
17d						+++++	++		

The number of “+” indicates the expression intensity of GAL4 in certain subset of MB neurons. This table is adapted from a previous study (Aso et al., 2009) with permission from the authors.