Spatio-temporal patterns of dCREB2 activity and long-term memory formation in *Drosophila*

By

Anne K. Tanenhaus

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The dissertation is approved by the following members of the Final Oral Committee:
Jerry C.P. Yin, Professor, Medical Genetics and Neurology
Peter Lipton, Professor, Neuroscience
Kate O'Conner-Giles, Assistant Professor, Genetics and Molecular Biology
Avtar Roopra, Associate Professor, Neuroscience
Antony Stretton, Professor, Zoology
Brian Baldo, Assistant Professor, Psychiatry

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Abstract

A key strategy for mediating cellular change is the ability to adopt a new repertoire of gene expression. As in other adaptive processes, memory formation requires the recruitment of new gene expression, and the transcription factor CREB (cAMP response element binding protein) is a key mediator of this process. However, CREB is also involved in a number of other cellular processes including metabolic regulation, circadian rhythmicity, and regulation of intrinsic excitability. For the first part of my dissertation work, we developed a new reporter system in order to create an integrated picture of the spatio-temporal pattern of CREB across the brain. This reporter approach allows us to continuously measure *Drosophila* CREB (dCREB2) activity in specific subsets of neurons in freely moving animals. Using this reporter, we found that dCREB2 activity undergoes circadian oscillations across the nervous system, including in brain areas that support memory formation. Applying this approach to different transcription factors, we found that circadian oscillations in transcription are common in the brain. For the second part of my dissertation, we used this reporter to examine late stages of dCREB2 activity during long-term memory consolidation. We found that in a specific subset of neurons in the ellipsoid body of the central complex, dCREB2 activity is elevated for multiple days after spaced training. This elevation specific to long-term memory, and is modulated by training strength. In the mushroom body, we found that dCREB2 activity is initially suppressed in some subsets of neurons, and elevated in others. After 12-24 hours, dCREB2 activity is periodically elevated in several subsets of neurons, suggesting ongoing maintenance. These observations provide insight into

mechanisms of systems consolidation in *Drosophila*, and generate new predictions about the diverse roles of CREB in memory formation. For the third part of my dissertation, we begin to characterize the phenomenology of memory enhancement, in order to ask which factors modulate the threshold for memory formation.

Published Materials

The following paper and manuscript are reproduced as published or as submitted within the text:

Tanenhaus AK*, Zhang J*, Yin JCP (2012) In vivo circadian oscillation of dCREB2 and NF-κB in the *Drosophila* nervous system. *PLoS ONE* 7:e45130. **equal contributing co-authors* (Chapter 2)

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Data from the following published material have been included, but the discussion of these data has been modified:

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Chapter 1: Introduction

Diverse Roles of CREB in Memory Formation

A fundamental feature of the brain is its ability to adapt. Neuroplasticity occurs in many contexts: during development, in response to injury, and in response to diet and drugs. However, one of the most remarkable examples of neuroplasticity is the ability of a nervous system to adapt to learned experience. This set of adaptations, in sum, constitutes the process of memory formation.

Throughout biology, a key strategy for mediating cellular change is the ability to adopt a new repertoire of gene expression. Transcription factors are central regulators of gene expression, and are an integral step in conveying cellular inputs into cellular responses. Like other adaptive processes, memory formation recruits new gene expression. The core component of this is the communication of synaptic activity into the recruitment of new gene expression to support the stabilization of synaptic changes. However, as with any cell, neurons must also negotiate other inputs beyond synaptic activity, and must make other adaptations beyond synaptic stabilization. Measuring the global picture of transcriptional processing provides a more holistic view of memory formation. The transcription factor CREB (cAMP response element binding protein) is a central mediator of long-term memory formation. CREB is broadly conserved throughout the animal kingdom, and CREB-dependent gene expression plays a role in a wide variety of cellular processes. These processes include cell survival, proliferation, differentiation, metabolic regulation, circadian oscillations, synaptic plasticity, and regulation of intrinsic excitability.

Memory formation is also shaped by a broad repertoire of systems-level processes. Among these are mechanisms of systems consolidation: transfer, reorganization of neural circuits, reactivation, and modulatory inputs from a variety of circuits. In addition to this, a number of other biological processes feed into the formation and maintenance of memory. Notably, circadian mechanisms are central to the maintenance of long-term memory (reviewed below). The brain must also negotiate memory formation with a host of other homeostatic processes, such as sleep and metabolic regulation. CREB is involved in a number of these processes: CREB plays roles in sleep homeostasis, circadian regulation, and in regulation of memory formation by starvation. CREB is also capable of shaping systems-level encoding by shaping the cellular allocation of memories (see section iv).

As a field, neuroscience has made enormous strides in understanding both the molecular and systems-level mechanisms of memory formation. As a result of reductionist approaches, we now understand an enormous amount about many mechanisms in neurobiology. We have come a long way in identifying and characterizing the players (molecules, cells), and mechanisms of how they interact (pathways, circuits). It would be naïve to assume that we have identified even the bulk of these components, and there are undoubtedly large advances to be made. However, one of the central challenges that emerges out of an abundance of information is the ability to integrate this information into an understandable ecology. For instance, how do the molecular changes in memory formation work within the systems biology of memory formation? There are several strategies for this: one approach is in

computational modeling. A biological strategy is to begin to map key molecular events into their natural context. This ability to make molecular observations *in vivo* is central both in integrating molecular, systems, and behavioral mechanisms of memory formation, and in integrating conventionally memory-related processes with other brain functions.

In this thesis, I describe a reporter tool that I have developed in *Drosophila*, and use this tool to make observations about dCREB2 activity over the course of normal circadian and sleep-wake cycles, and during later stages of memory processing. These observations inform our understanding of broader roles of CREB activity in memory processing. In addition, in a behavioral study using a courtship memory paradigm I begin to explore the phenomenology of memory enhancement, with the goal of helping to understand new roles for CREB in memory formation.

Background

CREB activity is governed by numerous regulatory mechanisms

CREB is a member of the bZIP transcription factor superfamily. In mammals, CREB and its close relatives CREM, and ATF-1 comprise the CREB family of transcription factors, likely derived from a single precursor. In *Drosophila* there is a single homolog for the CREB/CREM family of transcription factors, dCREB2. All CREB genes are characterized by the same set of structural features (**Figure 1.1**). A conserved basic region/leucine zipper (bZIP) domain is located near the carboxy-terminal, which mediates dimerization, and binds cAMP-response element (CRE) DNA sequences. CREB

family members can homodimerize or heterodimerize to bind CRE sites, which consist of the palindromic consensus sequence (3'TGACGTCA5') present in the promoter of a wide variety of genes. Sandwiched between two glutamine-rich domains (Q1 and Q2) is a conserved central kinase inducible domain (KID, or P-box), which contains phosphorylation sites for protein kinase A (PKA), and several other kinases, including protein kinase C (PKC), calcium/calmodulin-dependent kinases (CaMK) II and IV, mitogen/stress-activated kinase (MSK), MAPKAP kinase 2, AKT, and ribosomal S6 kinase (S6K), and casein kinase (Altarejos and Montminy, 2011; Horiuchi et al., 2004; Tubon and Yin, 2008).

The best studied among the CREB phosphorylation sites is the Ser-133 residue. Phosphorylation at Ser-133 is necessary for the association of CREB with the transcriptional co-activator CBP (or its paralogue, p300). CBP associates with the phosphorylated KID domain, and recruits transcriptional machinery to the CBP/CREB complex. Alternately, CREB can be activated by the TORC (Transducers of Regulated CREB activity) co-activator family, which also facilitates association of CREB with transcriptional machinery independent of Ser-133 phosphorylation. Several other phosphorylation sites have been identified as regulators of CREB activity. For example, the phosphorylation at Ser-142 has been reported to disrupt the interaction of CREB and CBP (Kornhauser et al., 2002; Radhakrishnan et al., 1997; Wu and McMurray, 2001). However, other studies indicate that activity-dependent Ser-142 phosphorylation positively regulates CREB activity (Gau et al., 2002). Additionally, phosphorylation at Ser-133 and Ser-142 induces phosphorylation by casein kinase II (CKII) at Ser-143

(Dudek, 2007). *In vitro*, kinase activity can lead to the phosphorylation of a dozen other serine residues, though the relevance of these sites *in vivo* is unknown (Johannessen et al., 2004). CREB can be inactivated as a result of dephosphorylation, primarily by protein phosphatase 1 and 2A, which can also be activated by PKA activity, or by calcium-respsonsive signaling cascades (Johannessen et al., 2004). Aside from phosphorylation, several other posttranslational regulatory mechanisms have been reported to modulate CREB, including glycosylation, sumoylation, acetylation, and ubiquitination (Sakamoto et al., 2011). Because of these features, CREB is a convergent target of a variety of activity-dependent signaling cascades, which can be triggered by increases in intracellular calcium through ligand or voltage gated channels, increases in cAMP, and other signaling mechanisms.

In addition to these post-translational modifications, CRE-mediated gene transcription is also regulated by the competition between CRE-binding transcription factors, including other members of the CREB family and activator or repressor isoforms. At a translational level, CREB genes undergo extensive alternative splicing, which can result in transcriptional activator as well as transcriptional repressor isoforms of CREB protein ((Habener et al., 1995; Yin et al., 1995b; Bartsch et al., 1998; Mayr and Montminy, 2001). In *Drosophila,* the *dCREB2* gene contains seven exons, and several dCREB2 forms are generated by alternative splicing, or from C-terminal truncation. In addition, *dCREB2* contains multiple translation initiation sites, which lead to the expression of different dCREB2 activator or repressor variants (Tubon and Yin, 2008; Tubon et al., 2013). For all species studied, the expression and regulation of these

different isoforms is an important factor in the regulation of CRE-mediated transcriptional activity (Barco et al., 2008). In addition, examples of CREB regulation by microRNAs have also recently been reported (Balschun et al., 2003; Blendy et al., 1996; Hummler et al., 1994).

CREB can also be regulated at the transcriptional level. A notable example of this is the expression of the transcriptional repressor ICER (inducible cAMP early repressor), which is transcribed off of an alternate promoter (P2) in the CREM gene. ICER proteins contain the bZIP domain, and can therefore dimerize with other members of the CREB family, but lack transactivation and KID domains. Because the P2 promoter contains CRE sites, ICER expression is induced by CREB activity, and subsequently represses its own transcription (Borlikova and Endo, 2009; Mioduszewska et al., 2003). Interestingly, in addition to competing for CRE sites, ICER has been proposed to regulate CREB protein stability (Borlikova and Endo, 2009). However, in *Aplysia*, the CREB1 promoter itself contain active CRE sites, and have been shown to mediate sustained CREB activity over many hours, suggesting a positive feedback loop in CREB activity (Liu et al., 2008, 2011; Mohamed et al., 2005).

Several studies have also identified the cellular localization of CREB or CREB activators as regulators of CREB activity. Nuclear translocation of CREB plays a role in CREB activation in vascular smooth muscle cells (Stevenson et al., 2001). In hippocampal cultures, CREB is has been detected in dendrites, and can be synthesized and phosphorylated locally (Crino et al., 1998). In dorsal root ganglion cells, stimulation by nerve growth factor induces retrograde transport of CREB from the axon to the nucleus

(Cox et al., 2008). In hippocampal neurons, the CREB co-activator CRTC1 (TORC1) translocates from synapses to the nucleus in response to synaptic activation (Ch'ng et al., 2012). Importantly, in flies nuclear entry, rather than Ser-231 phosphorylation is likely a dominant regulatory step in gating dCREB2 activity, including during memory formation (Fropf et al., 2013; Horiuchi et al., 2004).

The ultimate regulation of CRE-dependent gene expression reflects the convergence of numerous signaling pathways, and is under the control of a multitude of regulatory mechanisms at the transcriptional, translational, and post-translational level. This places CREB downstream of a wide variety of different pathways that will be modulated in different ways by a number of different types of inputs. Because of these features, individual molecular events, such as Ser-133 (or Ser-231 in flies) phosphorylation provide an incomplete view of the ultimate activation state of CREB.

The reporter system described in this thesis measures CREB-dependent transcription based on the expression of an artificial luciferase transgene under the control of a CRE promoter element. This allows us to measure the end product of the ensemble of CREB regulatory signaling, providing a more global view of CREB activity. Furthermore, this convergence of signaling pathways on CREB means that a variety of different types of pathways that respond both to neuronal activity, and to other stimuli, are negotiated in regulating CREB activation. This thesis addresses focuses particularly on circadian regulation of CREB activity, which is discussed in more detail in Chapter 1.

CREB-dependent gene expression, in turn, plays a role in a number of cellular processes, including cell survival, proliferation, differentiation, metabolic regulation,

synaptic plasticity, and regulation of neural excitability. In this thesis, I do not discuss at length CREB-target genes, which are explored in detail elsewhere (Sands and Palmer, 2008; Zhang et al., 2005). However, thousands of genes contain CRE promoter elements, and CREB also regulates the expression of other transcriptional cascades. Particular genes that are regulated by CREB will also be under the control of other regulatory mechanisms that will gate the expression of those particular genes. Therefore, the types of cellular states that are governed by the repertoire of gene expression downstream of CREB will be selected or refined in different contexts. For the purposes of this thesis, we simplistically consider two types of inter-related CREB-dependent states: a state that makes cells more amenable to synaptic plasticity (eg. due to the expression synaptic plasticity-related proteins), and a state that makes cells more intrinsically excitable (eg. due to the expression of ion channels).

CREB is required for memory formation

A fundamental feature of long-term memory is its requirement for protein synthesis (Davis and Squire, 1984; Hernandez and Abel, 2008), and a large body of work has implicated CREB as one of its mediators. Subsequently, the specific requirement for CREB in memory formation has been demonstrated across several species.

In mice, early loss-of-function approaches initially yielded mixed results regarding the role of CREB in memory formation. The first generation of CREB knockout mouse resulted in a hypomorphic mutant deficient in the α and δ CREB isoforms. Initial experiments suggested long-term memory deficits (Bourtchuladze et

al., 1994), however these impairments are sensitive to genetic background and gene dosage (Gass et al., 1998; Graves et al., 2002). Subsequently, conditional knockout mice were generated using floxed CREB alleles. However, these mice also fail to exhibit consistent hippocampus-dependent memory deficits (Balschun et al., 2003). Likewise, CREB knockout mice show inconsistent performances in amygdala-dependent memory tasks (Bourtchuladze et al., 1994; Gass et al., 1998; Graves et al., 2002; Balschun et al., 2003). These early controversies regarding the requirement for CREB in memory formation are largely resolved, and initial mixed results are attributable to compensatory up-regulation of other CRE-binding factors, such as CREM (Balschun et al., 2003; Blendy et al., 1996; Hummler et al., 1994). Consistent with this, dominant negative approaches have reliably demonstrated the requirement for CREB in memory formation. For example, expression of the dominant negative KCREB in dorsal hippocampus impairs spatial memory (Pittenger et al., 2002), and hippocampal induction of a CREB repressor variant specifically impairs contextual fear conditioning and spatial memory (Kida et al., 2002; Kim et al., 2011).

In *Drosophila*, the requirement for dCREB2 in long-term memory formation is more immediately obvious, likely due to the consistent use of a dominant-negative approach. In flies, the induction of a dominant negative isoform of dCREB2 blocks long-term memory (LTM), but not short- or middle-term memory for aversive olfactory classical conditioning (Yin et al., 1994). dCREB2 is also required for courtship suppression memory (Sakai et al., 2004), appetitive classical conditioning (Krashes and Waddell, 2008), larval appetitive memory (Honjo and Furukubo-Tokunaga, 2005), and

for the generation of associative memory traces (Akalal et al., 2006). To date, aside from anesthesia-resistant memory (see section vii), no examples of consolidated memory have been reported in *Drosophila* that do not require dCREB2 activity.

Synaptic Plasticity, Intrinsic Excitability, and CREB's Role in Memory Formation

CREB Activity Promotes Long-Term Synaptic Plasticity

Prevailing models of memory formation indicate that long-term modulation of synaptic function underlies memory formation and stability. It is generally accepted that CREB is a key component in mediating the molecular consolidation of synaptic change. As with LTM, sustained forms of long-term potentiation (L-LTP) require protein synthesis. In *Aplysia*, CREB-mediated transcription represents a critical step in the formation of LTF, and the injection of CRE sequences in sensory neurons specifically blocks long-term facilitation (LTF) (Dash et al., 1990). The injection of phosphorylated ApCREB-1 itself is sufficient to induce LTF (Bartsch et al., 1998; Casadio et al., 1999), and blocking the repressor form of CREB (ApCREB-2) enhances LTF (Bartsch et al., 1995). Microinjection of anti-CREB antibodies, likewise, prevents LTF (Martin et al., 1997). This role for CREB in synaptic plasticity is conserved in mammals. In hippocampus, protocols that induce L-LTP also induce CREB phosphorylation, as well as CRE-*lacZ* reporter expression (Bito et al., 1996; Impey et al., 1996). Interestingly, non-L-LTP protocols that induce CREB phosphorylation do not always result in increases in CRE-reporter expression (Impey et al., 1996). Mirroring studies of behavioral memory, early loss-of-function approaches had mixed effects on LTP (Bourtchuladze et al., 1994; Gass

et al., 1998; Rammes et al., 2000; Balschun et al., 2003). However, dominant negative forms of CREB (KCREB, or ACREB) result in deficits in several forms of L-LTP (Pittenger et al., 2002; Huang et al., 2004; Jancic et al., 2009). Conversely, expression of the constitutively active VP16-CREB lowers the threshold for induction of L-LTP in CA1 neurons, and bypasses the requirement for new protein synthesis (Barco et al., 2002).

CREB also plays a role in synaptic morphological changes that support memory formation. In *Aplysia*, the formation of LTF is accompanied new synaptic growth, that occurs downstream of CREB activity (Kandel, 2001). In cultured cortical neurons, estradiol-induced dendritic spine formation requires CREB activation (Murphy and Segal, 1997). Similarly, viral expression of an activated form of CREB, CREBCA promotes the formation of silent synapses, and increases dendritic spine density in CA1 pyramidal neurons (Marie et al., 2005). Finally, in visual cortex, viral expression of a dominant-negative CREB form leads to a reduction in average spine head size (Suzuki et al., 2007).

Together, these studies indicate that CREB activity plays a critical role in the formation of sustained forms of LTP, and other forms of synaptic plasticity, and that the products of CRE-mediated gene expression are necessary and sufficient to support the stabilization of synaptic potentiation and synaptic growth.

CREB Activity Increases Intrinsic Excitability

Recent studies in mammals have identified an additional conserved role for CREB in the regulation of intrinsic excitability. Here, excitability is defined as the

overall propensity of the neuron to generate action potentials (Daoudal and Debanne, 2003). In medium spiny neurons of the nucleus accumbens (NAc), viral manipulation of CREB activity substantially affects intrinsic excitability (Dong et al., 2006). While the resting potential and action potential wave form are unaffected by CREB manipulation, expression of constitutively active CREB (caCREB, or $CREB^{Y134F}$) substantially increases spiking in response to depolarizing current injections, while expression of a dominant-negative CREB form (dnCREB, or $CREB^{S133A}$) decreases it. This effect is attributed to both an increase in potassium conductance, as well as a decrease in sodium conductance, though the specific molecules involved are still unidentified (Dong et al., 2006). In nucleus accumbens, long-term social isolation induces depression and anxiety-like symptoms (Wallace et al., 2009). Likewise, social isolation induces a decrease in neuronal excitability (as measured to the number of spikes in response to current injection) in the nucleus accumbens shell (NAcSh), and a decrease in CREB activity. This process is correlated with an increase in K^+ channel expression. Furthermore, activation of CREB activity blocks social isolation-induced anxiety symptoms (Wallace et al., 2009). These data are consistent with a model in which social isolation-induced reductions in CREB activity mediate a reduction in excitability in the NAcSh, leading to the anxiety-like symptoms of social isolation. In support of this model, overexpression of the K^+ channel Kir2.1 in the NAcSh induces anxiety-like (but not depression-like) symptoms (Wallace et al., 2009).

CREB, likewise, modulates intrinsic excitability in the neuroadrenergic neurons of the locus coeruleus (LC). Viral expression of a dominant negative CREB isoform

hyperpolarizes LC neurons, and decreases basal firing rate, while expression of a constitutively active CREB isoform depolarizes LC neurons, and increases firing rate (Han et al., 2006).

Similarly, CREB activity affects excitability in hippocampal CA1 neurons. The expression of the constitutively active VP16-CREB does not affect resting membrane properties, but dramatically increases the number of action potentials resulting from depolarizing current injection (Lopez de Armentia et al., 2007). These hippocampal changes in excitability also occur *in vivo* (Gruart et al., 2012). Conversely, mice expressing the dominant negative A-CREB show a reduction in excitability (Han et al., 2006; Cao et al., 2010). This decrease is likely to be at least partially mediated by changes in the afterhyperpolarization (AHP), as expression of VP16-CREB substantially decreases the amplitude of the slow and medium afterhyperpolarization (AHP) current, while A-CREB mice show an increase in the medium AHP (Lopez de Armentia et al., 2007; Jancic et al., 2009).

In the lateral amygdala, overexpression of CREB in certain cells favor the recruitment of those cells into new fear memory traces, while inhibition of CREB decreases the probability that a given cell will be recruited (Han et al., 2007). As with hippocampus, expression of VP16-CREB, or viral expression of CREB increases the intrinsic excitability of neurons in the amygdala, and reduces their after-hyperpolarization (AHP) (Viosca et al., 2009; Zhou et al., 2009). Furthermore, increasing neuronal excitability in a random subset of neurons in the amygdala either by expression of a dominant negative potassium channel, through the activation of

DREADD receptors, or by optogenetic stimulation will also favor the recruitment of these cells to memory traces. Importantly, any of these stimulations (increasing excitability, increasing CREB activity) delivered immediately before training will enhance memory formation.

Currently, it is unclear the exact mechanism by which CREB regulates excitability. In NAc, it seems that increases in excitability are due to both increases in Na^+ conductance and decreases in K^+ conductance. This may be due to increased transcription of the Na^+ channel 1β subunit, or reduction in Kv1.4 subunit expression (McClung and Nestler, 2003). In hippocampus, A-CREB mice also show an increased rheobase current and a reduction in membrane resistance that were rescued by M potassium channel blockers, suggesting that hippocampal AHP modulation may be due to an increase in M potassium current (Jancic et al., 2009). Other studies indicate that decreased CREB expression up-regulates several types of K^+ channels in NAc (Wallace et al., 2009). In hippocampus, likewise, decreased CREB activity appears to up-regulate M-type potassium channel activity (Jancic et al., 2009). The mechanism through which CREB transcription would promote suppression of potassium channel expression is unclear. As with synaptic roles for CREB activity, these changes in excitability state are likely to be mediated by a host of different genes, and may be the result of other transcriptional cascades that are under CREB control. More thorough identification of the CREB targets that regulate excitability will lend significant insight into these issues.

Neuronal excitability as both a driver *and* a read-out of neuronal activity

Putting all of these observations together, a coherent model might suggest that activation of CREB by neuronal activity facilitates both synaptic potentiation and intrinsic excitability. Synaptic potentiation then makes cells more likely to be activated within the specific pattern of connectivity that represents a memory (the engram), and intrinsic excitability makes cells more likely to be activated in general. Subsequent neuronal activity facilitated both by synaptic potentiation and excitability should, in turn, activate CREB activity (**Figure 1.1**).

CREB-mediated intrinsic excitability and memory formation

What is the role for CREB-dependent regulation of intrinsic excitability in memory formation? While there is strong evidence that supports the role for CREB in mediating adaptations that support synaptic stabilization, the idea of a second, meta-plastic role for CREB in modulating excitability is intuitively appealing. There are several possible functions for CREB-mediated excitability modulation in memory formation, all of which are potentially inter-related.

Allocation

One possibility is that state of CREB activity mediates the allocation of memory formation to particular subsets of neurons. This is best supported by the body of work out of the Josselyn lab (discussed above), demonstrating that enhancing either CREB

activity, or intrinsic excitability will bias the allocation of fear memory to particular subsets of neurons (Han et al., 2007, 2009; Josselyn et al., 2001a; Yiu et al., 2014).

This has several important implications. First, this work indicates that, at least in amygdala, which neurons out of a set of possible cells are ultimately recruited to support a memory trace is plastic. Second, this work demonstrates the capability of CREB, and CREB-mediated modulation of excitability to shape this allocation. While this work shows very convincingly that CREB activity *can* shape the allocation of memory, the major question that is left is what *does* happen during memory processing.

In natural situations, this could have several possible functions. For instance, CREB activity could be recruited in a particular subset of cells by strong activity during memory formation. Subsequently, new information would be more likely to be recruited to these particular cells, due to their increased intrinsic excitability. This could play a role in binding relevant information to this particular circuit – a sort of cellular tagging. One important question that this raises is time course. For instance, CREB-dependent modulation of excitability could occur very acutely, and help to shape the formation of an engram during the early stages of memory formation. Another possibility is that CREB-dependent modulation of excitability could occur over a longer time course, and help to shape the incorporation of future information into existing memory circuits. This could occur by two mechanisms: one is through the persistence of CREB-target genes that are acutely activated during initial memory formation. Another possibility is that CREB activity is up-regulated, and is maintained in a more active state either through intracellular mechanisms, systems processing, or both.

All of these questions could be addressed by real-time observation of the pattern of CREB activity during, and following memory formation. In this thesis, I develop an approach to making real time measurements of CREB activity that lend insight into these issues.

Engram Maintenance

Another possible role for CREB-mediated excitability changes could be in maintenance of an existing memory circuit. If cells are more excitable, they will be more likely to be re-activated, further reinforcing synaptic potentiation of these circuits. Indeed, several learning and memory tasks produce sustained changes in intrinsic excitability, and regulate the size of the AHP (Moyer et al., 2000; Oh et al., 2003; Zhang and Linden, 2003; Saar and Barkai, 2009). It is unknown whether these changes are mediated by CREB activity, but learning-dependent long-term AHP regulation in CA1 neurons is mediated by PKA (Oh et al., 2009). Consistent with this, at least in early stages, AHP reduction is protein synthesis-dependent (Cohen-Matsliah et al., 2010). Likewise, long-term (24h) increases in excitability following operant conditioning in the *Aplysia* neuron B51, and this requires cAMP signaling at late stages (Mozzachiodi et al., 2008). Similarly long-term (24-48h) increases in both LTF and excitability are generated by serotonin treatment in *Aplysia* sensorimotor cultures, and these also require CREB at late states (Liu et al., 2008, 2011).

This type of role could be especially relevant in sleep and circadian-mediated memory consolidation. While circadian rhythms are generated and synchronized by

core timekeepers in the brain, circadian oscillations occur in peripheral tissues, and across the brain (Brown and Azzi, 2013; Eckel-Mahan and Storm, 2009; Górska-Andrzejak, 2013; Rath et al., 2014; Silver and Kriegsfeld, 2014), and circadian disruption is detrimental to memory maintenance (Eckel-Mahan and Storm, 2009). Most notably, circadian oscillations in cAMP and MAPK activity occur in hippocampal tissue. Inhibiting these oscillations by blocking MAPK activity after contextual fear conditioning, during daytime peaks in MAPK activity (but not during troughs) blocks behavioral performance measured days later (Eckel-Mahan et al., 2008). These data imply that circadian oscillations in this pathway are required for the maintenance of long-term memory. While CREB activity has not been tested directly in this context, given that its upstream pathway (cAMP/MAPK) is oscillatory, one appealing possibility is that CREB-mediated circadian oscillations in neuronal excitability facilitate memory consolidation, perhaps encouraging the reactivation of those particular ensembles of neurons in the memory trace during sleep (Skaggs and McNaughton, 1996). Consistent with this, in *Drosophila* dCREB2 activity is oscillatory in memory-related areas in the fly brain (Fropf et al., 2013; Tanenhaus et al., 2012, reviewed in Ch1 of this thesis).

How could a role in maintenance map onto the pattern of CREB activation during memory formation? This model is most consistent with a more long-lasting role for CREB-dependent gene expression during memory formation. One possibility is that CREB is activated during initial memory formation in a certain subset of neurons, and is maintained in an active state for a period of time by positive feedback or other regulatory mechanisms. This may be the case in *Aplysia*. Another possibility is that

CREB is reactivated within this particular memory circuit, and that this reactivation is coordinated during sleep or at particular circadian time points. The work described in this thesis lends support to both of these possibilities.

Gating

A third possible view for CREB-mediated changes in excitability is in gating memory formation by shaping the pattern of neuronal activation during systems-level memory processing. Relatively high levels of CREB activity should increase neuronal activity overall. This might facilitate memory formation in general, or might inhibit memory formation in general by facilitating inhibitory processes. Conversely, relatively low levels of CREB activity, resulting in relatively low excitability might facilitate memory formation in general through dis-inhibition, or might inhibit memory formation in general by raising the threshold for synaptic potentiation. Over the temporal domain, this type of activity could help to organize memory formation with other physiological constraints (for instance sleep-wake cycles or stages in systems processing). Over the spatial domain, this process might facilitate memory allocation, either between cells within a brain structure, or between particular brain structures (for instance transfer from hippocampus to cortex). The work presented in Chapter 2 suggest a potential gating role for CREB activity in systems-level memory processing.

Importantly, none of these three meta-plastic roles for CREB activity are mutually exclusive, and are probably interrelated. Together, they raise several questions

that form the framework for this thesis. There are two broad strategies for making sense of meta-plastic and excitability-related roles for CREB activity. The first is to observe the behavior of CREB within an intact system. The second is to attempt to isolate an excitability-specific role for CREB that is independent of its role in synaptic plasticity.

Late phases of CREB activation during long-term memory

While CREB activity is traditionally regarded as playing a role in memory by activating *de novo* gene expression as an immediate response to training, meta-plastic roles suggest that CREB might be recruited during other times. Across species, there have been observations of windows of time, post-training that are sensitive to protein synthesis inhibitors (Epstein et al., 2003; Grecksch and Matthies, 1980; Igaz et al., 2002). Several studies that have examined the time course of CREB activation specifically, after training have suggested a biphasic response. A second wave of CREB phosphorylation occurs in response to LTP induction in rat hippocampus, starting 2h following induction, and lasting for at least 24h (Schulz et al., 1999). Likewise, following long-term memory training in the Morris water maze, CREB phosphorylation in CA1 is biphasic, peaking immediately after training, and at again around 9h after training. This elevated phosphorylation lasts at least 24h, and is accompanied by downstream gene activation. The duration of the late phase of phosphorylation correlates with memory performance (Porte et al., 2008). In rats, inhibitory avoidance training leads to an initial increase in CREB phosphorylation in dorsal hippocampus, followed by a second wave of

phosphorylation 3-6 hours post-training, during a time that cAMP/PKA activity is required for memory consolidation (Bernabeu et al., 1997). In mouse, a similar biphasic pattern of activation has been observed in hippocampus, amygdala, and parietal cortex, with the second peak in CREB phosphorylation beginning between 3-6 hours after fear conditioning (Stanciu et al., 2001). Another group also found an increase CREB phosphorylation in hippocampus following inhibitory avoidance training, lasting at least 20h (Taubenfeld et al., 1999, 2001).

Finally, several studies in *Aplysia* find that CREB protein level and activity following induction of long-term facilitation (LTF) are active in two phases: a short-term increase that drops to baseline after 12h, and a later increase lasting at least 24h, accompanied by long-term CREB-dependent elevation in excitability (Liu et al., 2008, 2011).

While these studies provide clear indications of multiple waves of CREB recruitment during memory formation, there are a number of limitations to our understanding of the profile of CREB activity. First, these studies do not address time periods beyond 24h. Therefore, currently, the activation profile of CREB beyond 24h is not known. In addition, the majority of these studies use phospho-CREB antibodies to quantify the level of Ser-133 phosphorylation as a measure for CREB activity. However, due to the complex nature of the regulatory machinery for CREB (see section i), this is likely an incomplete view of the ultimate transcriptional activity pattern of CREB. Also, because it is impossible to continuously monitor CREB activity within the same population of animals, these approaches also all rely on serial time point measurements

among different animals, and cannot provide a coherent picture of the pattern of CREB activity over time and space. In this thesis, I address these limitations by developing a luciferase reporter system to measure CRE-dependent gene expression in defined anatomical subsets of cells. This new tool provides a general measurement of *Drosophila* dCREB2 activity, without relying on phosphorylation measurements. Furthermore, CRE-mediated gene expression can be measured in cell populations relevant to memory formation continuously, within the same freely moving animals. Because of this, dCREB2 activity can be monitored in real time for several days beyond initial memory formation, and within a systems context.

Questions:

1. How do patterns of CREB activity map into an *in vivo* context?
    - How is CREB activity organized over time across the brain? (Chapter 2)
    - How is CREB activity modulated by memory formation over time, and across the brain? (Chapter 3)
2. What mechanisms underlie CREB-mediated memory enhancement, and why does it only work sometimes? (Chapter 4)

The first two chapters of this thesis explore questions about the role of CREB activity that are observational in nature. First, what is the spatio-temporal pattern of CREB activity *in vivo*? Second, what is the spatio-temporal pattern of CREB activity during memory formation? These issues are addressed by making real-time observations of the state of CREB activity to inform our understanding of the role of

CREB activity in a natural context. The third chapter of this thesis is more artificial in nature, and is the beginning of an attempt to explore meta-plastic roles in memory formation by asking which inputs are capable of producing memory enhancement, and if they interact with CREB.

Figures and Legends

Figure 1.1

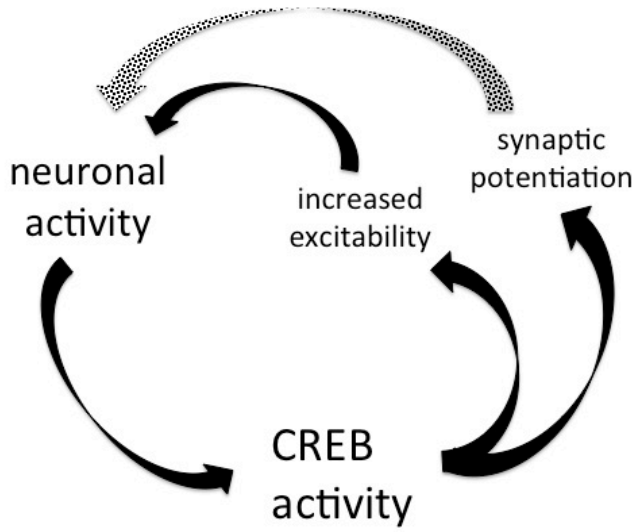

Figure 1.1: Positive feedback loop for CREB activity. Cartoon represents a hypothesized feedback loop for CREB activity. Increased CREB activity increases intrinsic excitability, and facilitates synaptic potentiation. Increased excitability leads to an increase in neuronal activity due to general inputs, while synaptic potentiation leads to specific patterns of neuronal activity, both of which then increase the probability of CREB reactivation.

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Chapter 2

A reporter strategy to measure transcriptional activity in targeted cell populations *in vivo* reveals widespread circadian oscillations in transcription

2.1 Introduction

Throughout evolution, behavioral and physiological processes have been coordinated to a 24h cycle. Even in the absence of inputs, individual cells are capable of maintaining a 24h cycle through interlocking molecular feedback loops. While it is becoming clear that any cell is capable of maintaining independent circadian rhythmicity, in complex organisms master timekeeping is generally delegated to particular neurons and brain areas (core clock neurons), which coordinate and synchronize rhythmicity in other cells throughout the body. CREB is generally recognized as playing a key role in mediating many features of circadian rhythmicity, but is largely studied only in core clock neurons.

While CREB activity is regulated by a variety of stimuli, the global pattern of CRE-driven activity in *Drosophila* is circadian. *In vivo*, the activity of dCREB2 can be measured in flies carrying a luciferase reporter gene under the control of multiple CRE elements (CRE-luc) (**Figure 2.1.1**) (Belvin et al., 1999). In this assay, luminescent activity can be measured in real time in live flies, maintained in 96-well plates on food containing the substrate luciferin (Brandes et al., 1996). CRE-luc flies show a robust pattern of reporter activity, that oscillates in a 24-hour rhythm with nighttime and daytime peaks in activity (Belvin et al., 1999). Reporter activity is drastically reduced in mCRE-luc reporter flies, in which the CRE binding sites are altered by two amino acids, and in the dCREB2 mutant S162 flies, indicating reporter specificity. These oscillations in

CRE-luc reporter activity are downstream of the molecular clock, as mutations in *period* modulate dCREB2 reporter activity rhythms (Belvin et al., 1999).

While there are clear connections between circadian rhythms and memory formation (Gerstner and Yin, 2010), it is unclear whether the circadian features of CREB activity are interrelated with other functions of CREB. Of particular interest is the role for CREB activity in memory and plasticity. For instance, CREB-dependent transcription may act within central and peripheral oscillators in mediating circadian functions, and play a separate role in other brain areas in memory formation. If this is the case, synchronous oscillations in dCREB2 activity from clock neurons may be responsible for the measured oscillations in CRE-luc reporter activity, drowning out other asynchronous dCREB2 activity.

A major limitation of previously existing luciferase-based transcription factor reporters is that specific cells or tissues cannot be measured independently without dissecting and culturing these tissues. This makes it very difficult to study rhythmic features of specific anatomical regions within their natural context. While global oscillations are can be detected *in vivo* using the CRE-luc reproter, it is unclear which anatomical regions generate these oscillations. There are several possibilities: 1) dCREB2 reporter cycling could be dominated by circadian oscillations from within a certain area, 2) dCREB2 reporter cycling may reflect the sum of different patterns of activity across different areas, 3) dCREB2 activity cycling is ubiquitous.

In order to ask which cell populations participate in circadian oscillations in dCREB2 activity, we sought to adapt the original CRE-luc reporter so that we could

isolate reporter signal to particular cells in the nervous system (Section 2.2). Because the same reporter design is applicable to any transcription factor response element, we applied this same approach to several other transcription factor families, identifying oscillations in other transcription factors, including nuclear factor kappa-B (NF-κB), hepatocyte nuclear factor 4 (HNF-4), and Cap'n'Collar (CNC). Section 2.2 describes the new, spatially delimited reporter system, and characterizes the circadian pattern of CREB and NF-κB activity in subsets of cells in the nervous system. Section 2.3 further characterizes the circadian pattern of activity in various neuronal subsets. Section 2.4 describes 24hr oscillations in HNF-4 activity identified using this reporter system.

Figures and Legends

Figure 2.1.1

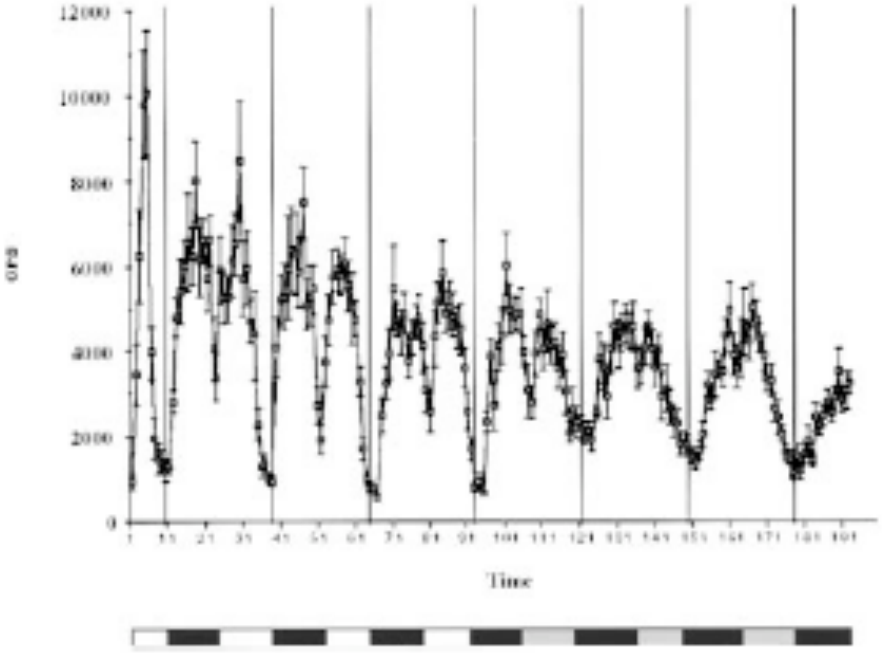

Figure 2.1.1: CRE-luc reporter activity in vivo. (Belvin et al., 1999). CRE-luc reporter activity oscillates in a 24h rhythm, which persists in constant darkness. Mean bioluminescence ± SEM is plotted over time. Bioluminescence is indicated relative light units, with counts per second (CPS) plotted. Light conditions (white bars), dark conditions (black bars), and subjective day (grey bars) are indicated below the graph.

2.2: *In vivo* circadian oscillation of dCREB2 and NF-κB activity in the *Drosophila* nervous system

Anne K. Tanenhaus*, Jiabin Zhang*, and Jerry C.P. Yin

* Authors contributed equally to this work

This section is a reproduction of a paper that has been published in its current form, except that references and figure headings have been adapted to the format for this dissertation. Supplementary materials published with this paper are included in Appendix A.

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Abstract

cAMP response element-binding protein (CREB) and nuclear factor kappa-B (NF-κB) are two ubiquitous transcription factors involved in a wide number of cellular processes, including the circadian system. Many previous studies on these factors use cellular assays that provide limited information on circadian activity or anatomical specificity. The ability to study transcription factors in defined tissue within intact animals will help to bridge the gap between cellular and *in vivo* data. We have used the GAL4-UAS and FLP-FRT systems to gain spatial control over reporter gene expression. Using a luciferase-based reporter, we show *in vivo* that *Drosophila* dCREB2- and NF-κB-mediated transcription oscillates in neuronal cells, glia, and in the mushroom body, a higher-order brain center in flies. This oscillation is under circadian control, cycling with a 24-hour rhythm, under both light-dark and dark-dark conditions. In light-light

conditions, dCREB2 and NF-κB reporter flies exhibit a suppression of rhythmic activity. Furthermore, neuronal cycling of dCREB2 and NF-κB activity are modulated in *period* mutant flies, indicating these oscillations are controlled through the central clock. This study shows for the first time region-specific circadian oscillation of dCREB2/NF-κB activity in the *Drosophila* nervous system.

Introduction

The biological importance of circadian rhythms is firmly established and widely accepted. The daily oscillation of internal function and behavioral output are present throughout evolution, and are conserved from invertebrates to humans. This circadian activity is self-sustaining, and can be synchronized to the local environment. Disruption of these rhythms has detrimental effects on health and cognitive function (Froy, 2011; Reid et al., 2011).

Cell-autonomous molecular oscillators govern circadian rhythms. In flies, the central pacemaker consists of a network of molecular interactions in a small population of cells. At the core of this machinery is a transcriptional feedback loop: the heterodimer CLOCK/CYCLE (CLK/CYC) activates the transcription of *period* (*per*) and *timeless* (*tim*). Once accumulation of cytoplasmic PER and TIM proteins is sufficient, they enter the nucleus and inhibit CLK/CYC, inhibiting transcriptional activity. The presence of these molecular oscillators in a subset of clock cells is sufficient to drive rhythmic responses throughout the organism (Allada and Chung, 2010).

Circadian changes in the expression of a large number of genes have been identified across many tissues in *Drosophila* (Boothroyd et al., 2007; Ceriani et al., 2002; Claridge-Chang et al., 2001; Keegan et al., 2007; Lin et al., 2002; McDonald and Rosbash, 2001; Ueda et al., 2002; Wijnen et al., 2006). While all of these studies identify transcripts with circadian expression patterns, there is often little overlap in the specific genes that are identified (Boothroyd and Young, 2008). Identifying transcription factors that are under circadian regulation could provide a means of simplifying the interpretation of this data. However, whether most transcription factors have oscillations in their activities is unknown. In this study, we focus on two ubiquitous transcription factor families that have been linked to the circadian system: cAMP response element-binding protein (CREB) and nuclear factor kappa-B (NF-κB).

The activity of the *Drosophila* CREB homolog (dCREB2) is under circadian regulation, oscillating in a 24-hour rhythm with nighttime and daytime peaks in activity (Belvin et al., 1999). Circadian control of CREB activity has important physiological implications. For example, CREB mediates the circadian modulation of gluconeogenesis in mice (Zhang et al., 2010). There is also evidence that CREB acts upstream of the circadian system. In the suprachiasmatic nucleus (SCN), the master circadian pacemaker in mammals, CREB plays a role in photic entrainment (Gau et al., 2002; Ginty et al., 1993; Lee et al., 2010; Obrietan et al., 1999; Tischkau et al., 2003) [15-19]. Suppression of CREB in the SCN inhibits expression of PER1 and PER2 (Lee et al., 2010). Likewise, a mutation in dCREB2 shortens the 24-hour circadian rhythm in flies, and impairs the oscillation of *per* (Belvin et al., 1999). Recently, cAMP signaling has been

demonstrated as an integral component of rhythmicity in the SCN (Atkinson et al., 2011; Doi et al., 2011; O'Neill et al., 2008).

There are similar indications that NF-κB interacts with the circadian system in mammals. NF-κB is present in the hamster SCN, and inhibition of NF-κB blocks light-induced phase shifts (Marpegan et al., 2004). Circadian variation in NF-κB activity has also been detected in the rat pineal gland (Cecon et al., 2010). Finally, effects of the circadian clock on apoptosis are mediated by NF-κB activity in mouse cancer models (Lee and Sancar, 2011). In *Drosophila*, the effects of the immune response on sleep are under circadian control, and may require the NF-κB family member *Relish* (Kuo et al., 2010).

Luciferase-based transcription factor reporters have provided significant insight into the function and regulation of the molecular clock (Asai et al., 2001; Brandes et al., 1996; Stanewsky et al., 1997; Wilsbacher et al., 2002; Yamaguchi et al., 2003; Yoo et al., 2004). Because luciferase activity is short-lived (Ignowski and Schaffer, 2004), and can be measured in behaving animals over extended periods of time, these reporters are powerful tools for measuring changes in molecular activities *in vivo*. Despite the advantages of this approach, it has rarely been applied outside of the core molecular clock to measure the general activity of transcription factors over the day:night cycle. We previously used a luciferase-based reporter gene under the control of dCREB2 binding sites (CRE-luc) to show that dCREB2 activity cycles (Belvin et al., 1999). An earlier report of NF-κB activity in flies utilized a reporter similar to the CRE-luc reporter, but it was not measured across the day/night cycle (Kuo et al., 2010). Until now, the major

disadvantage of this approach has been the inability to refine these measurements to specific tissues *in vivo*.

To gain spatial control of CRE-luc reporter activity, we take advantage of the UAS-GAL4 (Brand and Perrimon, 1993) and FLP-FRT (Golic and Lindquist, 1989) systems to restrict luciferase expression to specific populations of cells. We use the same strategy to generate a reporter fly based on a consensus sequence for NF-κB. In this study, we provide evidence *in vivo* that both dCREB2 and NF-κB transcriptional activities oscillate in multiple subsets of cells in the *Drosophila* nervous system. We show, furthermore, that these oscillations are under circadian control, and are modulated in *per* mutant flies.

Results

We generated modified versions of the CRE-luc reporter (Belvin et al., 1999) to measure transcriptional activity *in vivo* in defined subsets of cells. A reporter transgene was placed under the control of the FLP-FRT and GAL-UAS systems to allow for spatial control over reporter expression (**Figure 2.2.1 A**). The new reporters contain three binding sites for dCREB2 (or NF-κB) upstream of the CaSpeR TATAA element. Downstream of where transcription initiates, a Cavener sequence and translation initiation codon are positioned 5′ to an FRT-flanked cassette containing the coding sequence for mCherry (but without its normal start codon). The coding region terminates with two tandem stop codons. Translation that initiates at the ATG codon reads through the first FRT site and the mCherry sequence before terminating at the

tandem stop codons. The open reading frame for firefly luciferase (without its normal start codon) is placed downstream of the cassette. In the absence of FLP, translation should terminate at the tandem stop codons just 3′ to the mCherry sequence. In the presence of FLP, site-specific recombination between the FRT sites should remove most of the cassette, leaving one FRT site. Translation initiated at the first ATG codon should then read through the remaining FRT sequence, a poly-glycine encoding linker, and into the luciferase sequence, producing a fusion protein with luminescent activity. As a result, specific reporter expression should be activated when FLP recombinase is expressed under the control of an anatomically defined GAL4 driver.

To test if FLP recombinase can activate luciferase expression, we combined the CRE-F-luc transgene (**Figure 2.2.1 A #1**) with a heat-shock inducible transgene expressing FLP recombinase, and measured luciferase activity over time in live flies **(Figure 2.2.1 B)**. While flies carrying the CRE-F-luc transgene alone show no detectible luminescence, flies carrying an additional *hs*-FLP transgene exhibit substantial leaky reporter expression when maintained at 22°C. This activity increases significantly when these flies are exposed to heat shock. Similar to the previous reporter (Belvin et al., 1999), the CRE-F-luc reporter shows an oscillating pattern of day-night activity, characterized by daytime and nightime peaks. Light output dampens over time with luciferin degradation.

While FLP-dependence for reporter activation is important, the real utility of this system requires that GAL4-UAS mediated spatial control can be layered onto the reporter. In order to test this, we combined the CRE-F-luc transgene with a UAS-driven

FLP recombinase transgene (Duffy et al., 1998). Baseline expression in live UAS-FLP/+;CRE-F-luc/+ flies is detectable but low (~100 RLU/hr), and does not significantly increase in *hs*-GAl4/UAS-FLP/CRE-F-luc flies in the absence of heat shock (**Figure 2.2.1 C**). However, luciferase activity increases significantly in these triply transgenic flies following heat shock. Therefore, FLP recombinase expression under UAS control activates the reporter.

To verify the specificity of the fully assembled reporter, UAS-FLP;CRE-F-luc flies were crossed to an eye specific driver line, gmr^{long}-GAL4. Luciferase activity was then measured from isolated fly heads. This activity shows a substantial gmr^{long}-driver-dependent increase. However, there is no significant difference between groups when isolated bodies are measured (**Figure 2.2.1 D**), indicating specific reporter expression. Measurements of dissected head and eye tissue confirm that the signal is produced only in the eye tissue (**Appendix A : Figure S1**).

In order to characterize the range of signal detection, and to assess the contribution of various cell populations to circadian oscillations in dCREB2 activity, we screened a number of GAL4 driver lines for reporter activity. Flies containing both the UAS-FLP and CRE-F-luc transgenes were crossed to different GAL4 driver lines, or to wild type flies as a baseline control. In most of the lines tested, significant luciferase activity is detected above baseline (**Appendix A: Table S1**). A significant increase in reporter activity above baseline is detectible when GAL4 is driven in small populations of neurons in the ellipsoid body (*c42*-GAL4 or *c232*-GAL4) or in the mushroom body (MB, *ok107*-GAL4) (**Appendix A: Table S1; Figure 2.2.3 C)**, indicating that the reporter

can be used to measure activity in small subsets of cells. However, no signal is detectable using the *npf*-GAL4 driver (**Appendix A: Table S1**), which expresses in only a handful of cells(Lee et al., 2006). Notably, all drivers that produce detectible activity exhibit oscillating activity with the same general temporal pattern, peaking at around zeitgeber time (ZT) 4 (mid-day) and ZT16 (mid-evening).

We used the same strategy to build a reporter for general NF-κB activity. Our reporter is similar to a previous NF-κB luciferase reporter, except that it is FLP-dependent, and based on a more generalized NF-κB binding site (Kuo et al., 2010). As with the CRE-F-luc reporter, luciferase activity is undetectable in flies that contain only the NFκB-F-luc reporter, but FLP expression stimulates activity (**Figure 2.2.2 A**). When ubiquitously expressed under the control of an *actin*-GAL4 driver, NF-κB reporter activity oscillates in a 24hr rhythm under a light:dark regimen, peaking shortly after lights on (ZT1) (**Figure 2.2.2 B**). We observe a similar pattern when the reporter is activated ubiquitously by *hs*-FLP (**Appendix A: Figure S2**).

To verify that the NF-κB-F-luc reporter responds to NF-κB activity, we asked if established modulators of NF-κB would affect reporter activity. Because the innate immune response activates NF-κB (Dev et al., 2011; Kuo et al., 2010), bacterial infection should stimulate activity measured from NFκB-F-luc reporter flies. As the fat body is the center for innate immunity in *Drosophila*, we used a fat body-specific driver (*cg*-GAL4) to express the reporter in this tissue. These flies were inoculated with either a mixture of gram-negative and gram-positive bacteria, or received a sham injury. Bacterial infection, when compared to mock injection, significantly increases NFκB-F-luc

activity (**Figure 2.2.2 C,D**). This response peaks between 6 and 20 hours following infection, and lasts for about 30 hours.

To validate the specificity of the reporter, we asked if inhibiting NF-κB activity would decrease NFκB-F-luc activity. Pyrrolidine dithiocarbamate (PDTC) is a potent NF-κB inhibitor, suppressing NF-κB signaling via stabilization of I-κBα and inhibition of the ubiquitin-proteasome pathway (Bowie and O'Neill, 2000; Cuzzocrea et al., 2002; Gukovsky et al., 1998; Liu et al., 1999; Si et al., 2005; Virlos et al., 2003). Feeding of this drug has been used in *Drosophila* to suppress NF-κB activity (Moskalev and Shaposhnikov, 2011). Female UAS-FLP/+;NFκB-F-luc/*actin*-GAL4 flies were maintained for 24h on yeast paste containing PDTC and luciferin, and luciferase activity was measured 1h later *in vivo*. Reporter activity is reduced in a dose-dependent manner (**Figure 2.2.2E**). At the highest concentration (400mg/L), significant suppression of reporter activity lasts for 24h (**Appendix A: Figure S3**).

To measure the temporal pattern of dCREB2 and NF-κB activity in the nervous system, we activated the reporter under the control of the pan-neuronal *elav*^{c155}-GAL4 (**Figure 2.2.3A, E**), the pan-glial *repo*-Gal4 (**Figure 2.2.3 B,F**), or the MB-specific *ok107*-GAL4 drivers (**Figure 2.2.3 C,G**). For both reporters, we observe significant signal with each of the three drivers when compared to flies carrying the reporter and UAS-FLP transgenes alone (**Figure 2.2.3 D,H**). CRE-F-luc activity in neurons, glia, and the MB shows a similar diurnal pattern, with broad peaks during the middle of the day and the middle of the night (**Figure 2.2.3 A-C**). NF-κB reporter activity shows a similar oscillating pattern in neurons, glia, and the MB, with a major peak shortly after lights on

(**Figure 2.2.3 E-G**). Interestingly, unlike with the *actin*-GAL4 driver (**Figure 2.2.2 B**), a secondary peak in NF-κB reporter activity in the middle of the nighttime period is also present in neurons, glia, and the MB (**Figure 2.2.3 E-G**). In general, there is some variability in the presence of the secondary peak (data not shown), consistent with mixed reports of secondary peaks in *per* reporter activity (Brandes et al., 1996; Plautz, 1997; Stanewsky et al., 1997; Xu et al., 2008). This issue requires further investigation.

Because NF-κB reporter activity shows a robust rhythmic activity, we asked whether the reporter oscillates under circadian control. Robust oscillations in neurons, glia, and the MB are maintained in constant darkness after light:dark entrainment (**Figure 2.2.4 A**). Under LL conditions (see Methods), oscillations are maintained, but dampen over the course of light exposure (**Figure 2.2.4 B**). The persistence of oscillating activity in light-entrained flies maintained in constant darkness is strong behavioral evidence for circadian control of reporter activity. To determine if this activity is genetically under circadian control, we asked if mutations in the *period* gene (per^0, per^S and per^L) affect reporter activity. Flies were raised under a 12:12 light:dark cycle, and switched into constant darkness. As previously observed, neuronal NF-κB reporter activity is maintained under DD conditions in wild type flies (**Figure 2.2.4 C**). In contrast, NF-κB reporter activity in per^0 flies becomes arrhythmic (**Figure 2.2.3 D**), activity in per^S mutant flies shows a shorter period (~19 h, **Figure 2.2.3 E**), while per^L flies have an extended free-running period (~28h, **Figure 2.2.3 F**). In the original CRE-luc reporter, these mutations in *per* affected dCREB2 reporter activity in an almost identical manner (Belvin et al., 1999). dCREB2 reporter activity in neurons is similarly modulated

in per^0, per^S, and per^L mutants (**Appendix A: Figure S4**). Collectively, this data suggest that dCREB2 and NF-κB binding activity are likely to be under circadian control in most, if not all, nervous system tissues.

Because feeding behavior is also under circadian regulation (Xu et al., 2008), circadian changes in luminescence in dCREB2 and NF-κB reporter flies might be explained by diurnal variations in substrate availability due to feeding. To test this, we asked if oscillations in reporter activity persist after flies are removed from luciferin. UAS-FLP/+;CRE-F-luc/*actin*-GAL4 or UAS-FLP/+;NFκB-F-luc/*actin*-GAL4 reporter flies were kept on luciferin-containing food for 24 hours, and then transferred to 96-well plates containing non-luciferin food. Luminescence decayed rapidly, with a half-life of around 3.5-4 hours for the dCREB2 reporter flies, and 2.5 hours for the NF-κB reporter flies (**Figure 2.2.5**). However, oscillations in residual activity from both reporters are detectable for multiple days after substrate removal (**Figure 2.2.5** insets). A similar pattern of activity occurs for UAS-FLP/+;CRE-F-luc/*actin*-GAL4 flies whether maintained on, or removed from luciferin-containing food, with largely two peaks during the daytime and the nighttime (Fig. 1B, 3, 5A). Daytime and nighttime peaks of luciferase activity also persist beyond 24h after substrate removal in CRE-luc reporter flies (**Appendix A: Figure S5**). Likewise, UAS-FLP/+;NFκB-F-luc/*actin*-GAL4 reporter flies show the same general activity pattern both on and off of luciferin, with one dominant peak occurring shortly after lights-on (**Figure 2.2.2 B, 2.2.5**). Upon substrate removal, reporter activity patterns become more variable, perhaps attributable to increased variability of signal when luciferin is low, greater variability in residual substrate

between flies, or because substrate ingestion has some contribution to reporter activity patterns. Regardless, circadian variation in substrate feeding does not itself appear to explain circadian variation in reporter activity.

Discussion

We have used the FLP/FRT system to make spatially restricted transcription factor reporters. These transgenes contain reiterated consensus DNA binding sequences for single factors, thus providing a simpler, but cleaner picture of the activation state of specific transcription factors. The use of luciferase as the reporter moiety, which shortens the half-life of reporter activity, allows a more dynamic picture of transcription. This new reporter format produces detectable signal from behaving flies that is FLP-dependent (**Figure 2.2.1 B-C, 2.2.2 A; Appendix A: S1**). This allows the activity of specific factors to be measured in defined cell populations.

In general, the new CRE-F-luc reporter seems to be indistinguishable from the original CRE-luc reporter(Belvin et al., 1999). Both reporters show identical temporal patterns of activity, and *per* mutations modulate them indistinguishably (**Figure 2.2.1 B, 2.2.3 A-D; Appendix A: Figure S4**). When the CRE-F-luc reporter is expressed in certain small clusters of cells, activity cannot be measured, precluding us from concluding that dCREB2 activity cycles everywhere in the adult head **(Appendix A: Table S1**). However, since most major cell types and anatomical structures show oscillatory activity (**Figure 2.2.3 A-D; Appendix A: Table S1)**, we suspect that dCREB2's activity oscillates ubiquitously. This suggests that the experimentally observed oscillating activity does

not result from the activity of certain cells dominating the measured signal. Instead, the general diurnal pattern seems to occur when the reporter is activated in most, if not all, cells.

The NF-κB transcription factor reporter also oscillates, and is directly or indirectly under the control of the circadian system (**Figure 2.2.2 B, 2.2.3 E-H, 2.2.4**). The *Drosophila* genome encodes three NF-κB family members, characterized by a conserved DNA binding domain: Dorsal, DIF (dorsal-related immunity factor) and Relish. These proteins can form homodimers or heterodimers to regulate transcription. Because NFκB-F-luc reporter activity responds to known modulators of NF-κB activity (**Figure 2.2.2 C-E**), the oscillations we observe seem to reflect genuine changes in NF-κB transcriptional activity across the daytime and nighttime. However, we cannot discriminate which of the forms of NF-κB controls expression off of our reporter since Dorsal, Relish and Dif/Relish can bind to the consensus binding site used in our reporter fly (Senger et al., 2004).

Low-amplitude circadian fluctuations in luciferase reporter activity, even in predicted non-circadian reporters, are common(Stanewsky et al., 1997), though it is unclear if these rhythms are due to changes in transcription, or to non-transcriptional circadian changes that might affect luciferase activity (such as substrate feeding or locomotor activity). We can not definitively exclude the possibility that the oscillations in dCREB2 and NF-κB reporter activity are due to, or include, other non-transcriptional changes that are under circadian regulation. However, dCREB2 and NF-κB reporter oscillations do not appear to be explained by diurnal variations in substrate ingestion

(**Figure 2.2.5; Appendix A: Figure S5**). Furthermore, the relative changes in reporter activity that we observe are stronger than the low-amplitude changes (≤0.25 fold) reported in *hsp*-luc control reporters (Stanewsky et al., 1997), and in another transcription factor reporter that we have analyzed (data not shown). Finally, under light:dark conditions, the dCREB2 and NF-κB reporters show different kinetics, with the onset and peak of NF-κB activity (ZT≈1) occurring during a relative trough in dCREB2 activity (**Figure 2.2.1 B, 2.2.2 B, 2.2.3, Appendix A: Figure S2**). It seems most likely, therefore, that dCREB2 and NF-κB reporter oscillations represent genuine changes in transcriptional activity, rather than downstream artifacts.

We detect oscillatory dCREB2 and NF-κB reporter activity in neurons, glia, and the MB (**Figure 2.2.3**). While the specific roles of circadian activity in these particular tissues are unclear, oscillating transcription may be relevant to circadian-regulated functions in these areas. For example, a role for glial function in circadian behavior has recently emerged (Ng et al., 2011; Suh and Jackson, 2007). This raises the question of whether these oscillations in glia are required for any behavioral rhythmicity or if they modulate other physiological functions. In *Drosophila*, the MB is important for sleep regulation, a process that is an output of both circadian control and homeostatic drive (Joiner et al., 2006; Pitman et al., 2006). dCREB2 has been linked to the clock (Belvin et al., 1999), and has a non-circadian role in sleep-wake homeostasis (Hendricks et al., 2001). In addition, manipulation of PKA activity in MB affects sleep (Joiner et al., 2006). Further work is needed to determine if dCREB2 oscillation in the MB relates to the sleep-wake cycle. The MB is also an important learning and memory center. Although the role

of dCREB2 in the MB for olfactory long-term memory formation is controversial (Chen et al., 2012a), a long-term molecular memory trace may require dCREB2 in MB (Davis, 2011). Given recent mammalian data that suggest a role for circadian MAPK oscillations in memory maintenance (Eckel-Mahan et al., 2008), the possibility that dCREB2 activity cycling in the MB could be functionally important for memory processing is particularly intriguing. In mammals, NF-κB plays roles in both pathological conditions of neuronal disease (Pitman et al., 2006) and activity-dependent neural plasticity (Meffert and Baltimore, 2005). The functions of NF-κB in the *Drosophila* nervous system have not been extensively studied, although previous work shows that this family of transcription factors is important in immune function. Our NF-κB reporter fly provides another tool to study its function in both pathological and physiological conditions.

Our results suggest that the overall activities of these transcription factors have relatively simple diurnal patterns of activation *in vivo*, and that this is downstream of circadian oscillators. It is also commonly understood that both of these transcription factor families respond to a variety of acute and extra-cellular stimuli in cells. There are a number of non-mutually exclusive possibilities for how the overall patterns of activity that we observe could relate to acute responses that are occurring in the organism. First, there may be some stimuli *in vivo*, downstream of circadian activity, that are especially dominant during the mid-day and nighttime periods, producing peaks. The stimuli at these particular times may be targeted to most if not all cells, thus producing a strong signal. These possibilities are especially intriguing given that much of the reporter activity we observe occurs during time periods when flies typically sleep (Hendricks et

al., 2001; Shaw et al., 2000), a state characterized by neural synchronization in mammals (McCormick and Bal, 1997). Whether fly brain neurons show sleep-dependent synchronization is unknown, but there is some evidence that there are changes in neuronal activity during sleep (Nitz et al., 2002). How the patterned activity of these transcription factors relates to sleep state is an interesting question for future studies. Another possibility is that the other types of cues that can activate these transcription factors may be less global and more cell and tissue specific, thus producing limited activation. The resulting peaks in activation may be obscured beneath a background level of net circadian activity. Finally, dCREB2 and NF-κB may indeed respond to a plethora of signals, but *in vivo* these signals are synchronized by the circadian system to occur mostly during the two periods of peak activity. This last possibility implies that many physiological processes and their intercellular signals may be under circadian control in the animal, and occur at common times.

In this study, we focus primarily on the circadian pattern of dCREB2 and NF-κB activity in the nervous system. However, these factors are also involved in many other processes, including immune function (Dev et al., 2011; Wen et al., 2010), cancer (Perkins, 2012), aging (Zineldeen et al., 2010), neurodegenerative diseases (Mémet, 2006; Saura and Valero, 2011) and adult neurogenesis (Dworkin and Mantamadiotis, 2010). Since thousands of *Drosophila* GAL4 lines have been characterized, this technique provides a method to non-invasively measure dCREB2 and NF-kB transcriptional activity in multiple different tissues. We have also applied this reporter strategy to other transcription factors, which show different circadian and non-circadian patterns of

activity (data not shown). Therefore, we believe that this is a general approach that can be used to study transcription factors and their regulation in defined cell populations.

Materials and Methods

Fly Lines

The following fly stocks were used: *elav*-GAL4, UAS-FLP1, *hs*-FLP, *hs*-GAL4, gmr^{long}-GAL4, *actin*-GAL4 (Bloomington Stock Center), *ok107*-GAL4 (kindly shared by Y. Zhong), *repo*-GAL4 (kindly shared by B. Jones), $elav^{c155}$–GAL4 (kindly shared by K. Iijima-Ando), per^{0}, per^{S}, per^{L} (kindly shared by A. Sehgal), and *cg*-GAL4 (kindly shared by B. Ganetzky).

Reporter constructs

The new CRE-F-luc reporter contains three copies of the symmetrical CRE site (5'-TGACGTCA-3') situated upstream of a canonical TATAA element. A Cavener element (Cavener, 1987) (CAAC) and an ATG translation initiation codon are situated downstream of where transcription initiates, followed by a cassette containing FRT sites flanking the mCherry gene (without its own ATG start codon). Translation that initiates at the ATG codon would continue through the FRT site into mCherry, and would terminate at the end of the mCherry coding sequence. A short sequence coding for a poly-glycine run is downstream of the second FRT site, and placed so that it is in the same reading frame as the ATG start codon, regardless of which FRT site remains after site-specific recombination. The luciferase-coding region (minus its normal ATG start codon) is placed downstream, and in frame with, the poly-glycine run. In the absence of FLP, the transgene would produce a fusion protein with amino acids encoded by one FRT sequence, and the mCherry open reading frame. After FLP-mediated

recombination, a fusion protein would be encoded that contains amino acids from the FRT sequence and a poly-glycine run, all fused to luciferase. Sequence information is available upon request.

The NFκB-F-luc reporter relies upon a κB element (5′-GGGGACTTTCC-3′) contained in the mammalian immunoglobulin/HIV promoter (Van Gilst et al., 2005). The design of the transgene is identical to the CRE-F-luc, except that the kB element is substituted for the CRE sites. Reporter constructs were synthesized (GenScript), sequenced, and inserted at the NotI/XhoI sites of pCaSper5. Standard methods were used to generate transgenic flies (BestGene).

Luciferase Assays

In vitro biochemical assays of luciferase activity were preformed on frozen fly homogenates as previously described (Stebbins et al., 2001). For the head isolation experiment, three groups of 20 flies (10 male, 10 female) were frozen and heads and bodies were isolated and homogenized in cold homogenization buffer (15mM HEPES, 10mM KCl, 5mM $MgCl_2$, 0.1mM EDTA, 0.5mM EGTA) (Horiuchi et al., 2004). Luciferase activity was measured in triplicate using the SteadyGlo Luciferase Assay System (Promega).

In vivo luciferase assays were performed as previously described (Belvin et al., 1999; Brandes et al., 1996; Stanewsky et al., 1997). Briefly, 24-48 flies were entrained on a 12:12 light:dark cycle for 4-5 days and loaded into a black 96-well microplate containing luciferin media: 1% agar 5% sucrose food containing D-Luciferin (Gold BioTechnology).

5mM luciferin was used for most of the reporter assays: (**Figure 2.2.1, 2.2.3 A-D; Appendix A: Figure S2, Table S1**) or 25mM luciferin was used for all others. Plates were maintained at 22°C under controlled light conditions and cycled approximately once per hour through a Packard TopCount Scintillation and Luminescence counter. For all experiments, plates were in the dark during detection periods (~20 min/hr). The first 12-24 hours after loading are excluded from analysis to allow flies to recover from handling. A smoothing function is applied such that each data point represents the average of three measurements. For comparisons of overall activity between groups, the mean hourly reading is calculated for each fly and compared using Student's T-tests.

For pre-feeding experiments, flies were maintained in groups of 50 flies on media containing luciferin (100mM) for 24h before being loaded individually into 96-well microplates plates containing food alone (1% agar, 5% sucrose). The time delay between loading and the first luminescence measurement is between 30m and 1h.

Drug Feeding

Pyrrolidine dithiocarbamate (PDTC, Sigma) was mixed in yeast paste containing 15mM luciferin. Flies were maintained in standard food vials coated in yeast paste for 24h. After drug feeding, they were transferred to 96-well plates containing standard luciferin media for 1h before luciferase activity was measured.

Heat Shock

For heat shock experiments, flies were maintained at 22° throughout development. Flies receiving heat shock were placed at 37° (air) for 40 minutes. Flies were allowed to recover for 24h before all assays.

Bacterial Infection

Micrococcus luteus (gram-positive) and Escherichia coli (gram-negative) were cultured in LB medium overnight at 37°C. A mixture of these two types of bacteria was used for bacterial infection. A thin needle was dipped in a concentrated solution of bacteria and used to prick the thorax of adult flies (10~15-day old). Control flies received the same injury without bacteria. After infection, flies were loaded into a 96-well plate to monitor luciferase activity.

Figures and Legends

Figure 2.2.1

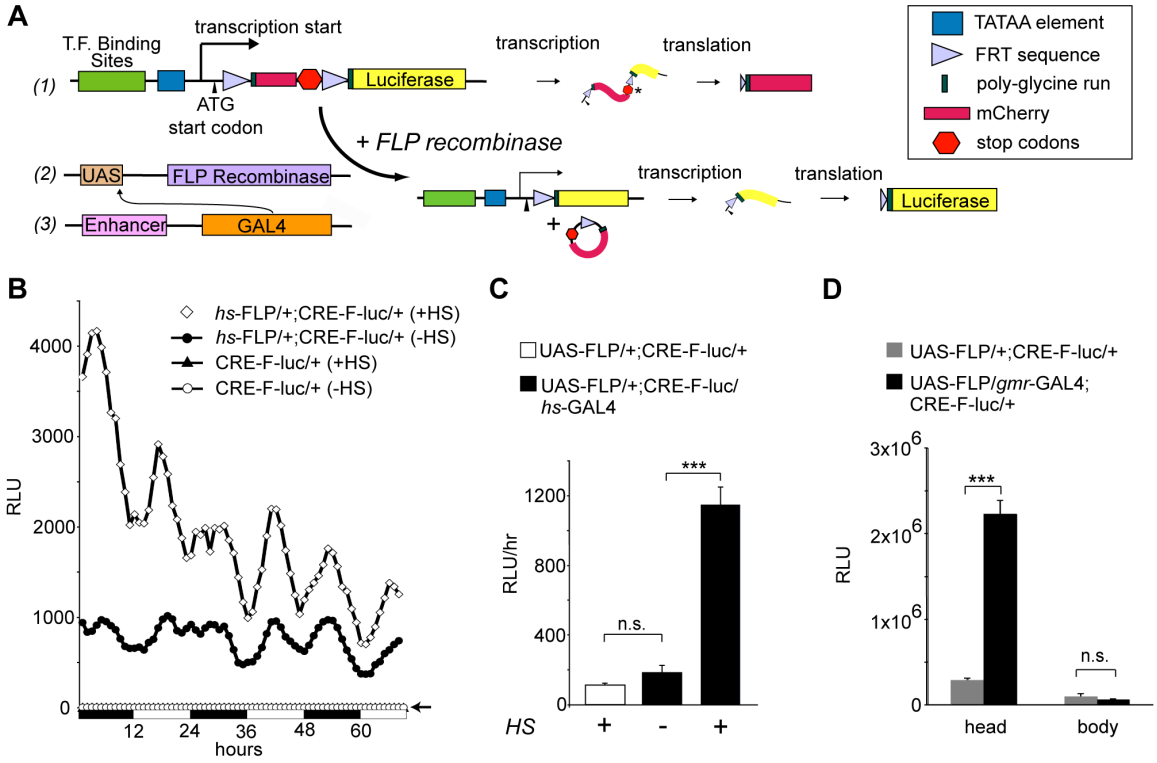

Figure 2.2.1. Developing a spatially restricted reporter system. (A) A cartoon of the reporter system. Three DNA binding sites are placed upstream of the CaSpeR TATAA sequence, followed by transcription (arrow) and translation (arrowhead) initiation sites, an FRT-flanked open reading frame for mCherry (ORF) including two tandem stop codons, and the luciferase ORF (#1). Targeted GAL4 expression (#3) drives the expression of UAS-FLP (#2), which catalyzes site-specific recombination at the FRT sites, activating the reporter. (B) CRE-F-luc reporter activity is FLP-dependent. Singly (CRE-F-luc) or doubly transgeneic flies (*hs*-FLP/+;CRE-F-luc/+) are maintained under 12:12 LD conditions. Flies are exposed to heat-shock (+HS) or not (-HS), and measured for *in vivo* luminescence. The relative luminescence is plotted as a function of time, with daytime (white bars) and nighttime (black bars) durations indicated below the graph. Each data point represents the average hourly luminescence counts in relative light units (RLU) of 24 flies. (C) FLP protein expressed using the GAL4-UAS system can activate the reporter. Reporter activity (Y-axis, the mean hourly relative light units [RLUs] over a 4-day window) is plotted as a function of genotype (indicated with different colored bars) and treatment (+/- HS, heat shock). Error bars = S.E.M. *** $p < 0.001$, Student' T-test, n=24; n.s. signifies not significant. Similar statistical comparisons are made for the remaining figures. (D) Anatomical specificity of gmr^{long}-GAL4 driven reporters. *In vitro* luciferase activity measured in extracts made from isolated heads and bodies. The relative light units (Y-axis) are plotted as a function of the genotype (shown in gray [UAS-FLP/+; CRE-F-luc/+ or black [UAS-FLP/gmr^{long}-GAL4; CRE-F-luc/+]) or tissue source (head versus body).

Figure 2.2.2

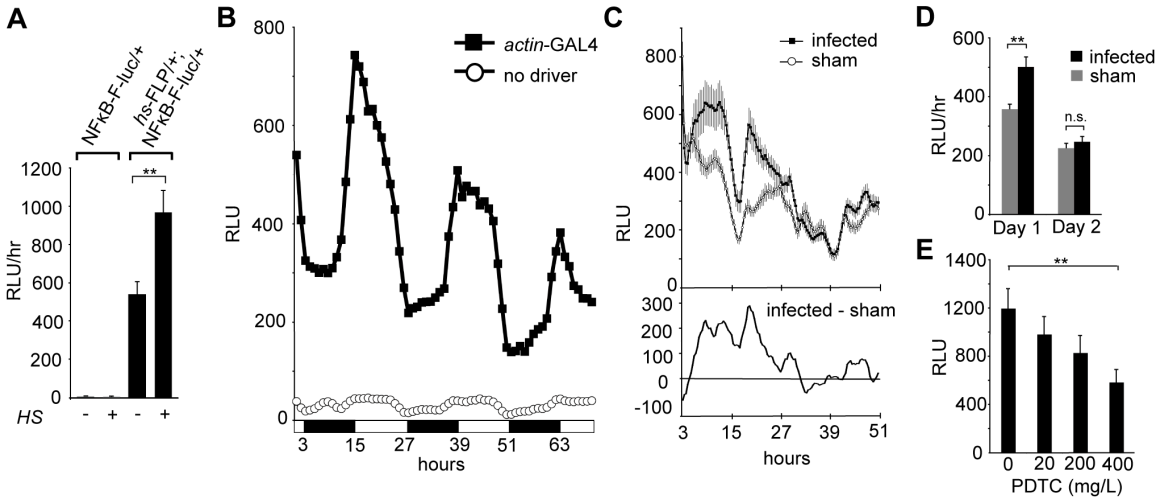
A
NFκB-F-luc/+
hs-FLP/+; NFκB-F-luc/+
RLU/hr
HS
B
actin-GAL4
no driver
RLU
hours
C
infected
sham
infected - sham
RLU
hours
D
infected
sham
RLU/hr
n.s.
Day 1
Day 2
E
RLU
PDTC (mg/L)

Figure 2.2.2. Expression of the NF-κB-F-luc reporter is FLP dependent, oscillatory and NF-κB-dependent. (A) NF-κB-F-luc reporter activity is FLP-dependent. Reporter activity (Y-axis, relative light units) is plotted as a function of genotype (singly [NFκB-F-luc] or doubly transgenic flies [hs-FLP/+;NFκB-F-luc/+] and treatment (heat shock HS+ or not, HS-). The histogram bars show the mean hourly counts over a 3-day window (n=24). (B) The ubiquitously expressed *actin*-GAL4 driver produces oscillatory NFκB-F-luc activity over a 24h period. Luciferase activity is plotted as a function of time for singly (denoted no driver; NF-κB-F-luc) or doubly transgenic (denoted *actin*-GAL4; NFκB-F-luc/*actin*-GAL4) flies. n=24 for each group (C) Bacterial infection affects reporter activity. Top panel: Reporter activity in the fat body is plotted for the first 48 hours after infection (filled circles) or sham treatment (open circles). The *cg*-GAL4 line (together with UAS-FLP and NFκB-F-luc) is used to activate the reporter in the fat body. n=48 for each group. Bottom panel: The difference in reporter activity between the infected and sham-treated groups (infected RLU-sham RLU) over the same duration post-injection. (D) Quantification of reporter activity (mean hourly count) during the first and second days following infection. The mean luminescence counts in RLU are plotted as a function of time after infection. Values for each day are averaged and binned together. (E) There is a dose-dependent decrease in reporter activity in flies fed PDTC. The relative luminescence is plotted as a function of the PDTC dose fed to flies. Triply transgenic flies (UAS-FLP/+;NF-κB-F-luc/*actin*-GAL4) were fed different dosages of PDTC for 24 hours and then measured for luminescence 1h after the end of feeding. n=24 for each group, Error bars = S.E.M, **p<.001.

Figure 2.2.3

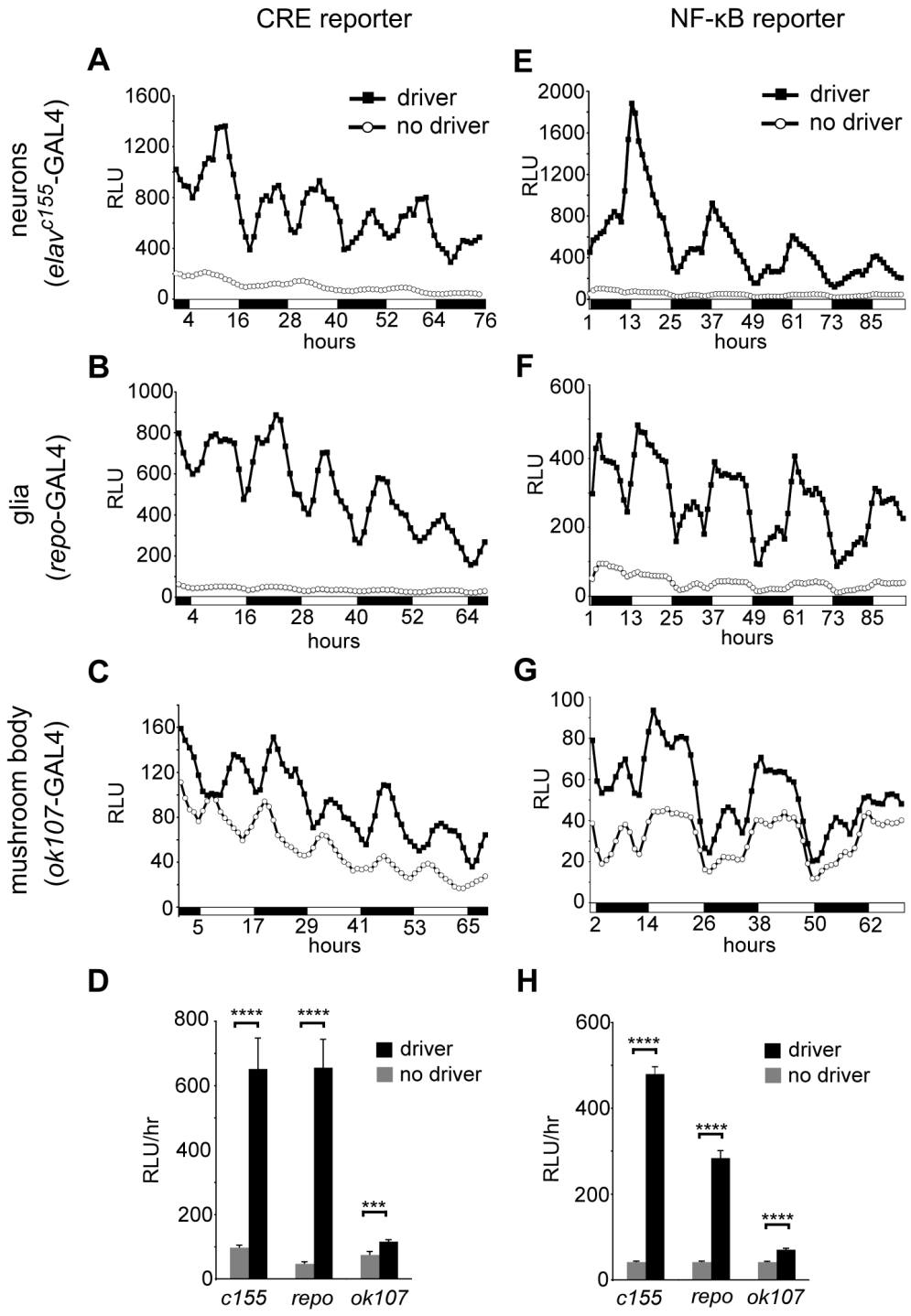
CRE reporter
NF-κB reporter
A
E
B
F
C
G
D
H
neurons (elav^c155-GAL4)
glia (repo-GAL4)
mushroom body (ok107-GAL4)
driver
no driver
RLU
hours
RLU/hr
c155
repo
ok107
****
***

Figure 2.2.3. dCREB2 and NF-κB activity cycle in multiple tissues. Reporter activity is plotted as a function of circadian time. Each different cellular population is described on the left, and the tissue-specific driver used to activate the reporter is shown. In each panel, a comparison is shown between the background (denoted no driver) and activated (driver) expression levels. The activity is shown for the CRE-F-luc (panels A-C) and the NFκB-F-luc (panels E-G) reporters in neurons (panels A and E), glia (panels B and F) and in the mushroom body (panels C and G). The average activity over the duration of the experiment for each driver is quantified for the CRE-F-luc (panel D) and NFκB-F-luc (panel H) reporters. All bars represent mean hourly counts from 24-28 flies. Error bars = S.E.M, ***p <0.001, **** p<0.0001.

Figure 2.2.4

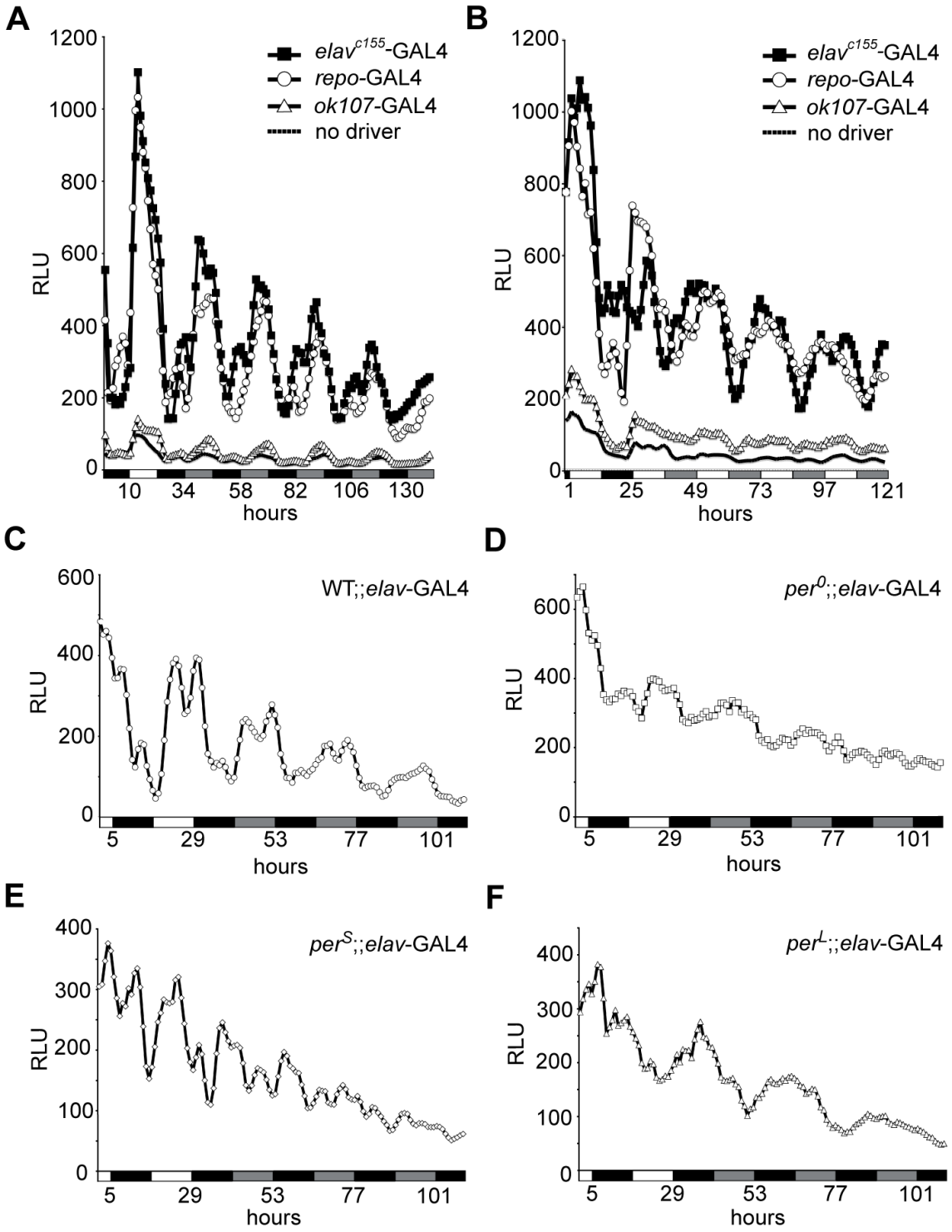
A
B
C
D
E
F
elav^c155-GAL4
repo-GAL4
ok107-GAL4
no driver
RLU
hours
WT;;elav-GAL4
per^0;;elav-GAL4
per^S;;elav-GAL4
per^L;;elav-GAL4

Figure 2.2.4. The NF-κB reporter activity oscillates under circadian control. (A) NF-κB reporter activity in multiple tissues oscillates in constant darkness. Reporter activity is plotted as a function of time for different genotypes. Triply transgenic flies, where the reporter is activated in all neurons (using the *elav*^{c155}-GAL4 driver, black squares), in a pan-glial pattern (using the *repo*-GAL4 driver, open circles), or in the mushroom body (using the *ok107*-GAL4 driver, open triangles), exhibit oscillatory activity when flies are maintained in light:dark (first 24h period) or dark:dark (subsequent time) conditions. These plots are compared to those from doubly transgenic flies (solid line; UAS-FLP/+; NFκB-F-luc/+) that do not contain the tissue-specific driver. (B) The activity of the NF-κB reporter in different tissues is dampened under constant light. Reporter activity is shown as a function of time, with the shift to constant light occurring during the second 24h period. The reporter is activated in triply transgenic flies in neurons (denoted *elav*^{c155}-GAL4, *elav*^{c155}-GAL4; UAS-FLP/+; NFκB-F-luc/+), glia (denoted *repo*-GAL4, UAS-FLP; NFκB-F-luc/*repo*-GAL4) or the mushroom body (denoted *ok107-GAL4*; UAS-FLP; NFκB-F-luc/+; *ok107*-GAL4/+) when compared to doubly transgenic flies without a GAL4 driver (solid line, UAS-FLP/+; NFκB-F-luc/+). (C-F) Neuronal reporter activity is plotted over time as flies are shifted from light:dark to constant darkness. For all of these panels, the same transgenes (UAS-FLP/+; NFκB-F-luc/*elav*-GAL4) exist in all flies, but the flies are examined in a wild type (C), *per*^{0} (D), *per*^{S} (E) or *per*^{L} (F) genetic background.

Figure 2.2.5

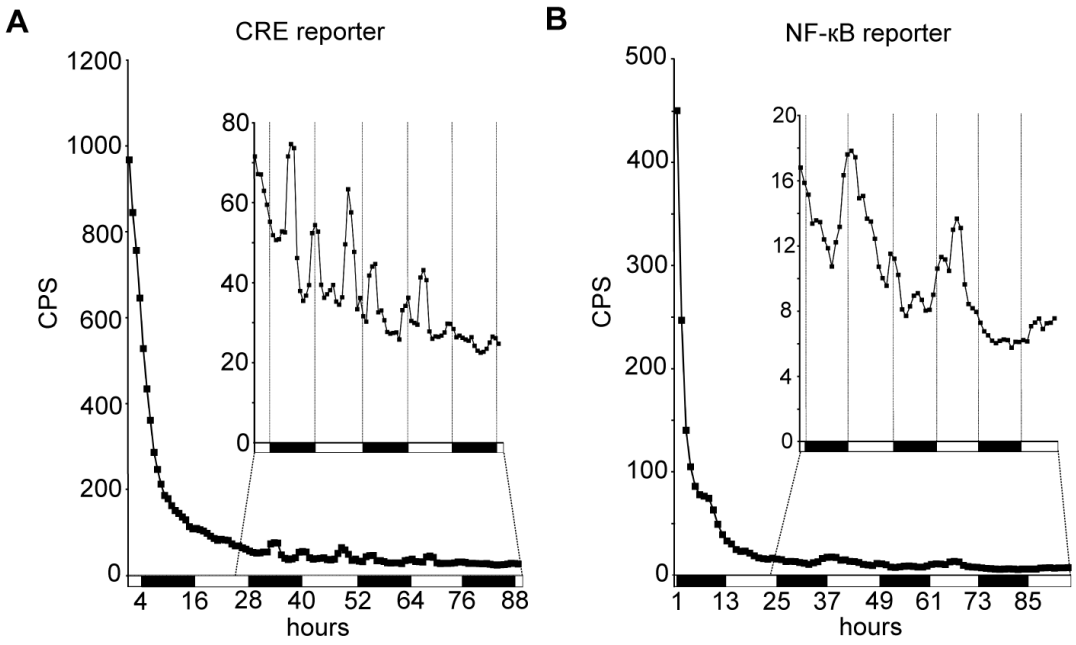
A
CRE reporter
CPS
1200
1000
800
600
400
200
0
80
60
40
20
0
4
16
28
40
52
64
76
88
hours
B
NF-κB reporter
CPS
500
400
300
200
100
0
20
16
12
8
4
0
1
13
25
37
49
61
73
85
hours

Figure 2.2.5. Oscillations in dCREB2 and NF-κB activity persist after luciferin removal. (A) Reporter activity in UAS-FLP/+;CRE-F-luc/*actin*-GAL4 flies starting ~1h following luciferin removal, plotted as a function of time. The inset shows a scaled view of the same data, with the first 24h dropped. (B) Reporter activity in UAS-FLP/+;NFκB-F-luc/*actin*-GAL4 flies starting ~1h following luciferin removal. Inset shows a scaled view of the same data, with the first 24h dropped. n=24 flies for each group.

2.3: Synchronous oscillations in dCREB2 activity across circadian and non-circadian cells in the nervous system

Data presented in this section (Figure 2.3.1) have been previously reported in:

Fropf R, Zhang J, Tanenhaus AK, Fropf WJ, Siefkes E, Yin JC (2014) Time of day influences memory formation and dCREB2 proteins in *Drosophila*. Front. Syst. Neurosci. (2014), 8:43

In the previous section, we report the CRE-F-luc reporter as a tool for measuring cell-specific luciferase activity under the control of the GAL4 system. We identify similar patterns of oscillations in many cell populations in the brain. Here, I sought to further characterize the pattern of dCREB2 activity across circadian and memory-related areas in the brain. One question was whether central clock neurons show distinct patterns of activity. Therefore, we measured CRE-F-luciferase activity under the control of several GAL4 driver lines in parallel. We chose driver lines encompassing the majority of clock neurons, as well as two central brain drivers that express GAL4 in the memory-related centers, the ellipsoid body (EB) of the central complex and the mushroom body (MB) (see **Table 2.3.1**). Because *cryp*-GAL4-16 and *tim*-GAL4 also express in glia, we also included the glia-specific GAL4 driver, *repo*-GAL4.

We found that dCREB2 activity show robust circadian oscillations in both clock and non-clock neurons, including glia, the central complex, and the mushroom body (**Figure 2.3.1 A**). This is pattern is consistent with the majority of other GAL4 drivers that we tested (Tanenhaus et al., 2012), with all cell populations showing broad mid-day and mid-night peaks in dCREB2 activity. Therefore, circadian modulation of CREB

seems to be a general feature among many different cells in the brain, and many different CREB-dependent mechanisms might be organized by the circadian clock. Furthermore, oscillations between clock and non-clock neurons are largely synchronous (**Figure 2.3.1, Appendix A: Figure S7**), suggesting that they might be modulated by similar mechanisms. Importantly, oscillations in dCREB2 activity occur in memory-related areas. This suggests that dCREB2 is oscillatory in the same cells in which dCREB2 would mediate memory formation. Therefore, it is possible that dCREB2 activity during memory formation may be influenced or synchronized by the circadian system.

Figures, Tables and Legends

Table 2.3.1

Driver Line	Expression pattern
c547	ellipsoid body (R2, R4m) neurons
ok107	mushroom body neurons
repo-GAL4	pan-glial expression
clk(Int-3)-GAL4	dorsal neuron clock cells
*cryp*GAL4-24 (cry24)	pdf neurons, dorsal neurons, ellipsoid body ring neurons, antennal neuropils
*cryp*GAL4-16 (cry16)	pdf neurons, ellipsoid body ring neurons, diffuse glial expression
tim-GAL4	all clock cells, several glia

Table 2.3.1: Expression pattern of driver lines. Circadian driver lines were characterized by (Kaneko et al., 2012; Zhao et al., 2003).

Figure 2.3.1

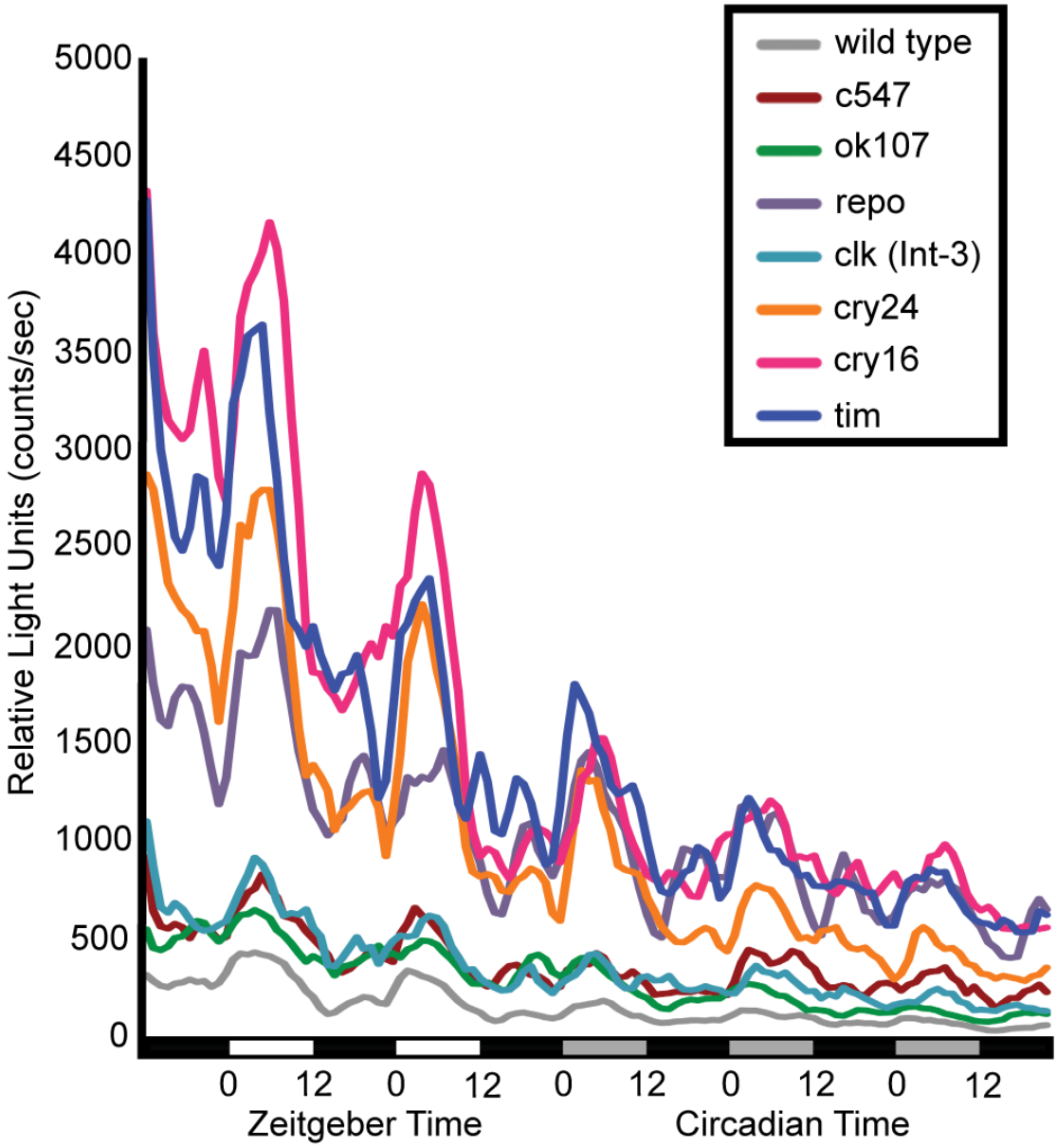
wild type
c547
ok107
repo
clk (Int-3)
cry24
cry16
tim
Relative Light Units (counts/sec)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
12
0
12
0
12
0
12
0
12
Zeitgeber Time
Circadian Time

Figure 2.3.1: dCREB2 transcriptional activity has a similar pattern of oscillation across cell populations. Bioluminescence is plotted in multiple fly lines expressing a dCREB2 luciferase reporter under the control of tissue-specific GAL4 drivers. Each line represents the mean reporter activity over time (hours) across flies (n = 48) representing the progeny of UAS-FLP;CRE-F-luc transgenic flies crossed with wild type (gray), or GAL4 driver lines: *c547* (red), *ok107* (green), *repo*-GAL4 (purple), *clk(int-3)-GAL4* (teal), *crypGAL4-24* (cry24, orange) *crypGAL4-16* (cry16, pink), *tim*-GAL4 (blue). Each data point represents the average over three hourly time points. Light conditions are indicated along the x-axis first in LD (black: night, white: day), then in DD (black: subjective night, gray: subjective day).

2.4: Diurnal rhythmicity in *Drosophila* HNF4 activity

Data presented in this section represent a portion of a manuscript in preparation:

Tanenhaus AK, Reed K, Tubon TC, Xing H, Yin JCP. Diurnal rhythmicity of dHNF4 activity in the *Drosophila* nervous system (in preparation)

Nuclear receptors are a large class of ligand-gated transcription factors that are involved in a wide variety of adaptive responses. Nuclear receptors generally act as sensors for lipid-soluble hormones and fatty acids, and are therefore well-positioned to coordinate metabolic responses (Chawla et al., 2001). Metabolic disorders, including diabetes are associated often with dysfunctions in nuclear receptor activity (Gat-Yablonski et al., 2006; Sonoda et al., 2008) and due to the interplay between metabolic and neuroinflammatory responses (Odegaard and Chawla, 2013), nuclear receptors have recently become a popular target in the development of clinical treatments for neurodegenerative disease (Skerrett et al., 2014).

Drosophila hepatocyte nuclear factor 4 (dHNF4) is a member of the nuclear hormone receptor family, the ortholog of mammalian nuclear receptors HNF4α and HNF4γ (Palanker et al., 2009). In flies, *dHNF4* subsumes the function of the mammalian peroxisome proliferator activated receptor (PPAR) and HNF4 families of nuclear receptors, and many of the identified dHNF4 target genes are orthologous to PPARα target genes (Van Gilst et al., 2005, Palanker et al., 2009). We sought to generate a reporter for dHNF4, based on multiple copies of the PPAR response element (PPRE) (5'-AGGTCA-3'). We first used EMSA analysis to ask whether dHNF4 associates with these binding sites. Using fly head extract, we found a gel shift species that is disrupted by

antibodies specific to dHNF4, and that does not form when a mutant probe is used, indicating that dHNF4 binds to these elements (**Figure 2.2.1 A**). Therefore, we refer to the binding sites for dHNF4 as HNF4 sites.

Changes in metabolism are a central feature of circadian rhythmicity, and a number of nuclear factors including PPARs show diurnally-regulated expression patterns, as do their target genes in mammals (Yang et al., 2006). Therefore, we generated a luciferase-based reporter transgene based on the CRE-F-luciferase design (see section 2.2) to measure HNF4 transcription factor activity in specific cell populations *in vivo*. This reporter is identical, except that multiple HNF4 elements are substituted for the CRE sites (HNF4-F-luc). To activate the reporter, we crossed HNF4-F-luc transgenic flies with flies carrying a heat-shock driven FLP recombinase (FLP) transgene. As expected, HNF4-F-luc is silent when FLP is not co-expressed, and expresses robust signal when hs-FLP is induced, that is attenuated in the absence of heat shock (**Figure 2.4.1 B, C**). These data confirm that like the CRE-F-luc and NFkB-F-luc reporters (see chapter 1.2), the HNF4-F-luc reporter is FLP-dependent. *In vivo*, HNF4-F-luc activity shows a complex diurnal rhythm, characterized by several peaks during the 24hr cycle: a peak early in the day, a peak near the light:dark transition, and a peak towards the end of the night (**Figure 2.4.1 B**). A spectral density estimation identified a dominant peak at a 24h frequency, further confirming the diurnal rhythmicity of reporter activity (**Figure 2.4.1 D**).

In *Drosophila,* dHNF4 highly expressed in a number of metabolic tissues, including gut, Malpighian tubules, and fat body, but is also expressed in the brain

(Palanker et al., 2009). Mammalian HNF4 is generally not expressed in brain tissue (Zhong et al., 1994), though brain expression has been reported all members of the PPAR family (α, β/γ, δ) . However, the role of dHNF4 in the brain has not been studied. Given the diurnal pattern of oscillation of HNF4 activity, we asked whether HNF4-F-luc reporter activity in the brain also oscillates. To test this, we expressed the HNF4 reporter under the control of enhancer-trap FLP lines (Fore et al., 2011) that produce pan-neuronal (232B) or pan-glial (777A) expression of FLP and measured dHNF4 activity in vitro. When the reporter is expressed in either neurons or glia, dHNF4 activity displayed a robust diurnal rhythm. Interestingly, neurons and glia showed different temporal patterns in reporter activity (**Figure 2.4.2**). While neurons showed relative peaks in the early morning (ZT= 1-2) and mid-evening (ZT=18), and troughs around lights-off (ZT=12) and late evening (ZT=21). Glia exhibited peaks at mid-day (ZT=7) and mid-evening (ZT=18), and troughs in the late day (ZT=10), and around lights-on (ZT=0). While we have not screened other tissues likely to recruit HNF4 activity, it appears that different cell types undergo distinct oscillation patterns, contributing to the complex global pattern of HNF4 activity across the entire animal (**Figure 2.4.1 B**).

These oscillations in HNF4 activity in the brain are particularly interesting, and may reflect distinct and coordinated metabolic-responsive processing in neuronal and glial populations. Fatty acid metabolism has been tied to several important nervous system functions. For instance, the mammalian fatty acid binding protein Fabp7 and its *Drosophila* homolog, dFabp both undergo diurnal changes in abundance in the brain and play an important role in sleep regulation (Gerstner et al., 2008, 2011). Determining the

significance of these time windows may provide insight into the role of dHNF4, and of fatty acid metabolism in the brain.

Circadian rhythm and sleep disorders are strongly associated with broad spectrum of metabolic symptoms (Maury et al., 2010; Turek et al., 2005), and therefore tools to study the pathways involved in the coupling between the circadian system and metabolic processing should provide useful insights that are relevant to human health. We hope that the HNF4-F-luc reporter will provide a useful contribution to these studies.

Methods

Electrophoretic mobility shift assays (EMSAs) were performed as previously described (Horiuchi et al., 2004). The sequence of the wild type probe contains four copies of the binding site for PPAR (dHNF4) (5'-AGGTCA-3'), while the mutant version contains changes to the conserved G nucleotides.

HNF4-F-luc reporter flies were generated as previously described (Tanenhaus et al., 2012), and are identical in design to these previously-reported FRT reporters except that multiple binding sites for the mammalian PPAR transcription factor family were used (5'-AGGTCA-3'). Enhancer-trap FLP lines 777A and 232B were kindly provided by T. Fore and B. Zhang.

In vitro luciferase assays were performed as previously described (Tanenhaus et al., 2012). Flies were entrained throughout development on a 12:12 light dark cycle that was maintained throughout the experiment, with the exception of intervals of time in the dark during bioluminescent measurements (<15m/hr). Flies were anesthetized (CO2) for loading onto luminometer plates, so for each experiment the first partial day was excluded from analysis to allow flies to recover from anesthesia.

Spectral density estimations were performed using R statistical analysis software. Raw means of the hs-FLP/+;HNF4-F-luc (+HS) time series were analyzed using the spectrum{"stats"} function (R Core Team, 2013).

Figures and Legends

Figure 2.4.1

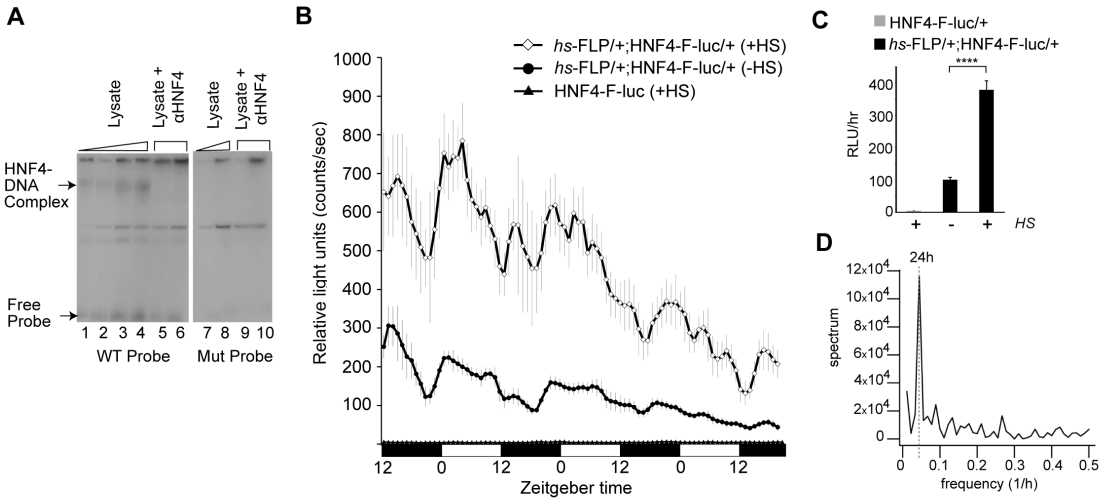

Figure 2.4.1. HNF4 reporter activity over the day-night cycle. (A) The *Drosophila* HNF4 protein specifically binds to the DNA sequences located in the HFN4 reporter. EMSA analysis was used to show that the binding activity in fly head extracts for the HNF4 sites contains dHNF4 protein. Radioactively labeled wild type (lanes 1-6) or mutant HNF4 sites were in EMSA experiments using fly head extracts as the protein source. When the wild type site is used as the probe, there is a retarded gel shift species whose intensity is a linear function of the amount of protein added to each reaction (Lanes 1-4). This species does not form over mutant HNF4 sites (lanes 7-10), showing that mutations that disrupt HNF4 binding preclude complex formation. The complex that forms contains dHNF4 protein, since the addition of dHNF4-specific antibody (lanes 5-6) disrupts the formation or mobility of the complex. (B) FLP-activated HNF4-F-luc activity. Singly (HNF4-F-luc) or doubly transgenic flies (*hs*-FLP/+; HNF4-F-luc/+) are maintained under 12:12 LD conditions. Flies are exposed to 37° 30m heat-shock (+HS) or not (-HS), and measured for *in vivo* luminescence. The relative luminescence is plotted as a function of time, with daytime (white bars) and nighttime (black bars) conditions indicated below the graph. Note: data for singly transgenic (HNF4-F-luc) flies are plotted near the x-axis. Each data point represents the average of 24 flies, and a smoothing function was applied such that each point represents a moving average of 3 data points. Error bars = S.E.M (unsmoothed) (C) Quantification of luciferase activity over the course of the experiment. Bars indicate mean hourly counts. (Error bars = S.E.M, **** = $p<0.0001$). (D) Periodogram of the raw mean data for the *hs*-FLP/+; HNF4-F-luc/+ (+HS) group. Spectrum value is plotted with respect to frequency (1/1h). The

dotted line indicates 24hr frequency, and the dominant peak located at ≈0.042 (24hrs) indicating a diurnal rhythmicity.

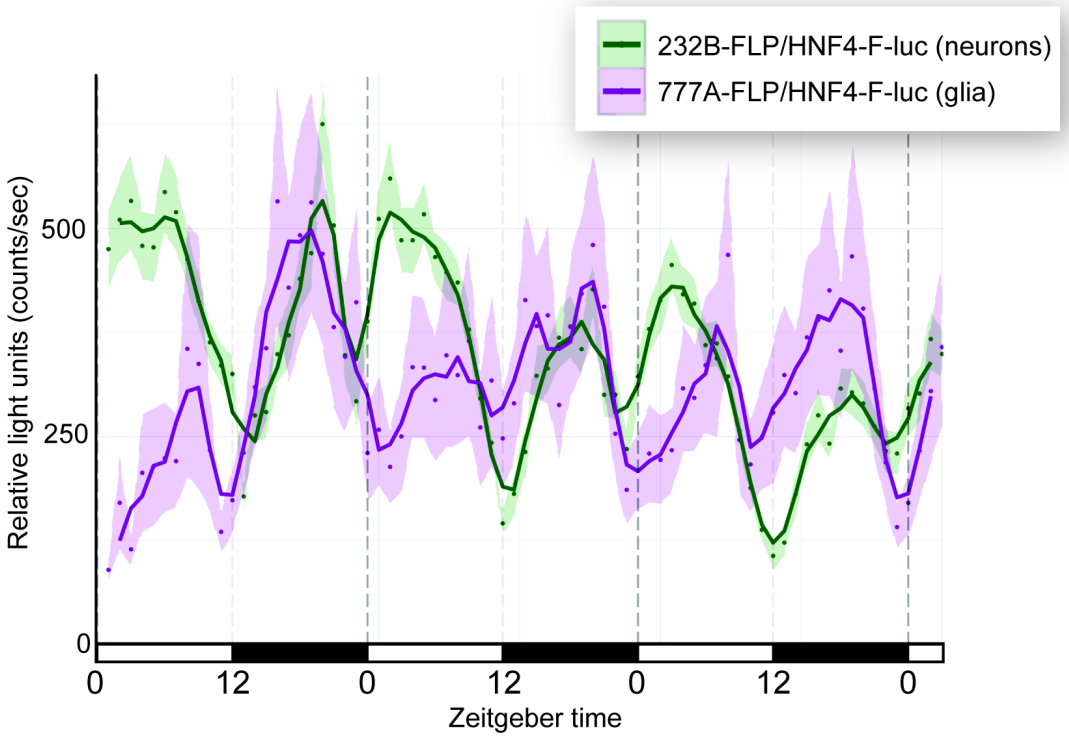
232B-FLP/HNF4-F-luc (neurons)
777A-FLP/HNF4-F-luc (glia)
Relative light units (counts/sec)
500
250
0
0
12
0
12
0
12
0
Zeitgeber time

Figure 2.4.2. Distinct patterns of HNF4-F-luc oscillations in neurons and glia. Bioluminescence plotted over time in doubly transgenic flies carrying the HNF4-F-luc reporter activated by an enhancer-trap flippase line: 232B (pan-neuronal, green) or 777A (pan-glial, purple). Flies were entrained throughout development under 12:12 light dark conditions. Light conditions are indicated along the x-axis (black: night, white: day). Points indicate raw means for each data point, and ribbons indicate the SEM. Lines indicate a moving average of 3 data points.

2.5 General Discussion

FRT-luciferase reporters are a general strategy to characterize transcription factor activity patterns

This chapter describes a new development of an approach for measuring tissue-specific transcription factor activity *in vivo*. This approach uses luciferase as a reporter molecule, which has several advantages over other genetically-encoded reporters. β-galactosidase is one of the first and most commonly used reporter molecules, but is unsuitable for *in vivo* imaging. Fluorescent molecules are optimized for imaging-based analysis, and have been extremely for both molecular and systems-level studies. However, this requires external delivery of an excitation wavelength, and outside of a slice or tissue culture system live imaging is generally limited to shallow tissues and/or immobilized animals. As a reporter molecule, luciferase has a number of distinct advantages over fluorescent or other enzymatic-based assays. Luciferase has a very short half-life of activity, making it well suited to measure dynamic changes. Importantly, luciferase activity can be detected at extremely low levels, both due to the detection capabilities of luminescence counters and to enzymatic signal amplification. Therefore, bioluminescent signal can be detected through the cuticle of a live fly, even from within subsets of cells. In these attributes were harnessed in pioneering work that developed an *in vivo* bioluminescent assay for *Drosophila* to describe *period* transcription (Brandes et al., 1996; Stanewsky et al., 1997). In this assay, substrate can be delivered easily through feeding, and within the confines of a 96-well plate individual flies can move freely during bioluminescent measurements.

Despite these advantages, real-time luciferase assays in live *Drosophila* have not been commonly used because they have been limited by their lack of anatomical specificity. However, currently tools for conditional expression are more thoroughly developed, and widely available in flies. By making these reporters FLP-dependent, they can be used to in combination with the wide variety of conditional expression tools.

In neuroscience, the ability to make molecular observations *in vivo* is an important step in integrating molecular and systems processes. This *in vivo* approach is particularly well suited to circadian observations, as transcriptional events can be monitored over several days, and under normal activity patterns.

While I have focused on circadian regulation of transcription factor activity, these reporters are broadly applicable for studies of many different processes, and have already been used both in and out of the circadian field (Choi et al., 2012; Kayser et al., 2014). Chapter 3 describes one application of the CRE-F-luciferase reporter to the study of spatio-temporal changes in dCREB2 activity in long-term memory processing.

Oscillations in transcription are widespread

One of the key observations from this chapter is how commonly we find circadian regulation of transcription, even outside of central clock tissues. In many ways this is not surprising- circadian rhythms occur throughout all branches of life, and all biological mechanisms will have evolved in conjunction with these circadian mechanisms. While the bulk of circadian research has focused on self-sustaining oscillatory cells, there is a great deal to be gained in the study of clock-controlled genes

and processes in other tissues, and in integrating our understanding of biological processes with their circadian patterns.

We have now applied our reporter strategy to target consensus sequences for five different classes of transcription factor: dCREB2 (Section 2.2, 2.3), NF-κB (Section 2.2), dHNF4 (Section 2.4), Cap'n'Collar (**Appendix A: Figure S6**), and the PAS Domain family (personal communication, R. Fropf). In all five of these reporters, we observe circadian or diurnal oscillations in reporter activity, most with distinct temporal patterns of activity. For the three factors for which I have characterized spatial patterns of activity, two appear to be largely synchronized across different tissues (dCREB2, NF-κB), and one appears to be asynchronous across different tissues (dHNF4). These observations raise several key questions:

1. What is the mechanism controlling these oscillations across tissues?
2. What is the general purpose for circadian-regulated transcriptional oscillation?

Determining the driving mechanism for these oscillations will require a good deal of future study, though in theory any number of different regulatory signals could account for the circadian patterns of oscillation (neuronal activity, metabolic signals, hormonal signals, internal circadian control). In the case of dCREB2 and NF-κB, organism-wide signals could provide one explanation. For example, melatonin circulates widely, is regulated by the central clock, and and regulates both dCREB2 and NF-κB (Carrillo-Vico et al., 2005; Cecon et al., 2010; McNulty et al., 1994). dHNF4 activity

which appears to be under circadian regulation, but has different temporal patterns in different tissues, likely reflecting tissue-specific modulatory or regulatory mechanisms. However, we tend to favor the general idea that the intracellular organizational principles in 'core' clock neurons are generally applicable to most cells: here, the global cellular landscape is organized by circadian-controled epigenetic, transcriptional, and translational mechanisms (Koike et al., 2012).

Perhaps the more interesting question is what the larger purpose for circadian-regulated transcription might be. Given the enormous complexity of regulatory mechanisms, signaling cascades, and other intracellular processes, there must be some organizing principles that coordinate these different cellular processes. In addition to spatial compartmentalization, temporal compartmentalization of subcellular processes allows incompatible subcellular processes (for example oxidative metabolism, reductive metabolism) to coexist (Tu and McKnight, 2006). Our results may have broad applications across a number of fields, and should be considered in many contexts, from basic research, to timing of drug delivery.

Acknowledgments: Chapter 2

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Chapter 3

Spatio-temporal dCREB2 dynamics in *Drosophila* LTM processing

3.1 Introduction

In the previous section I described a strategy to measure activity of transcription factors within subsets of cells in the fly *in vivo*. In this section, this strategy is applied after training to monitor real-time dCREB2 activity in different subsets of neurons that participate in systems-level memory processing in the fly brain. Using this information, I propose that dCREB2 activity is recruited during early and late stages of systems processing, and that this is likely to be interrelated with metaplastic roles for dCREB2. Furthermore, I integrate our results with existing models of systems processing in *Drosophila* to propose a hypothesis for a timeline that includes late changes in excitability and circadian-coordinated dCREB2 activity.

Background

Olfactory Avoidance Memory formation in Drosophila

The most commonly used behavioral paradigm for learning and memory in *Drosophila* is the olfactory avoidance task. In this form of classical conditioning, flies are trained to associate a particular odor (the conditioned stimulus), with a shock that is paired with that odor (the unconditioned stimulus). Olfactory memory is typically broken down into several phases, based on their duration and their differential genetic requirements: short-term memory (STM), middle-term memory (MTM), and two types of consolidated memory, protein synthesis-independent anesthesia resistant memory (ARM), and protein synthesis-dependent long-term memory (LTM). In this paradigm, a single training trial produces STM, while 10 training trials with intervening 15m rest

intervals (spaced training) produces LTM, lasting as long as 7 days. Ten-trial training with no rest intervals (massed training) leads to the formation of ARM, which can last as long as 4 days (Tully and Quinn, 1985), and is distinct both in its genetic requirements and its systems processing from LTM (Folkers et al., 2006; Knapek et al., 2011; Lee et al., 2011).

Anatomy and Circuitry of Olfactory Classical Conditioning

Mushroom Body

The mushroom body (MB) is the most established brain region involved in formation of olfactory memory. The MB itself consists of roughly 2500 Kenyon cells, whose cell bodies reside in a dorsal neuropil structure known as the calyx. These cells send processes towards the anterior brain via a bundle known as the peduncle. Most Kenyon cell axons diverge into two branches, with these axon branches forming dorsal (α,α′) and medial (β,β′) lobes. Another subset of cells sends projections that do not split, forming medial γ lobes (**Figure 3.1.1**). Within each of these lobes, there are anatomically-distinct sub-regions. The γ lobes can be subdivided into a main lobe, and a dorsal γ lobe, the α′β′ can be divided into anterior, middle, and posterior portions, and the αβ lobes can be subdivided into core, surface, and posterior portions. A number of GAL4 driver lines (generally enhancer trap lines) exist in *Drosophila* whose expression patterns map to different subpopulations of neurons within the mushroom body. These populations of cells are often preferentially located within particular lobes, or sub-lobes of the

mushroom body, and are often used to functionally dissect different subpopulations of cells within the mushroom body.

The MB is generally understood to be the critical area for olfactory memory formation. Chemical or genetic ablation of the MB abolishes olfactory memory (de Belle and Heisenberg, 1994; Heisenberg et al., 1985), and expression of a large number of memory-related genes are required in the MB for normal olfactory learning and memory (Kahsai and Zars, 2011). Within the MB, different subsets of cells mediate different types of memory processing, and different phases of memory processing over time (Dubnau and Chiang, 2013).

Ellipsoid Body

The ellipsoid body (EB) is located within the central complex of the fly brain. The central complex includes several structures: the fan-shaped body and surrounding superior arch, the EB, the noduli, and the protocerebral bridge. The cell bodies of EB neurons reside in the anterior rind. Their processes project to the lateral triangle, where they bifurcate before projecting towards the midline of the brain. Here, their processes form several concentric rings structures. EB neurons are generally classified by ring layer (termed R1, R2, R2, R4), and by the relative position of their cell bodies (medial, dorsal) (**Figure 3.2**).

The EB is not as well studied as the mushroom body in the context of olfactory learning and memory, though several studies have demonstrated a role for the EB in olfactory memory processing. Mutations that alter central complex structure are also deficient in olfactory memory (Heisenberg et al., 1985). Furthermore, NMDA receptors in the R2/R4m subset of neurons in the EB (but not in the MB, or in R3/R4d neurons), are required for olfactory LTM (Wu et al., 2007). This R2/R4m subpopulation of EB neurons is reciprocally connected to the MB, and has been shown to modulate olfactory MTM (Zhang et al., 2013b) . The R2/R4m neurons of the ellipsoid body are also important for adaptive responses to ethanol, for certain types of visual pattern memory, and for longer mating duration memory (LMD), suggesting that they may might play a modulatory role in multiple types of adaptive processing (Kim et al., 2012; Pan et al., 2009; Urizar et al., 2007). Though it is clear that the EB is involved in several memory-related tasks, and participates in olfactory memory circuits, the general role of the EB is still largely uncharacterized. Another subset of neurons that comprise the R3/R4d rings of the EB have no known role in olfactory memory.

Extrinsic Neurons

The mushroom body lobes also contain projections from several dozen extrinsic neurons, whose cell bodies reside outside the calyx. During early phases of memory processing, several of these cells have been identified that contribute to memory formation, likely as modulators of mushroom body activity: the anterior-paired lateral neurons (APL), the dorsal-medial paired neurons (DPM), and the dorsal anterior lateral

neurons (DAL). The bilateral pair of DPM neurons express a neuropeptide encoded by the *amnesiac* gene, and plays a critical role in middle-term memory (MTM) (Waddell et al., 2000). The APL and DPM neurons connect via heterotypic gap junctions within the α'β' lobes in the mushroom body, forming an apparently recurring circuit that stabilizes middle-term, anesthesia-sensitive memory (Pitman et al., 2011; Wu et al., 2011). The DAL neurons play a critical role in LTM formation, with inputs from the MB required during initial acquisition, and outputs to the mushroom body required during retrieval (Chen et al., 2012b).

Neurocircuitry of Aversive Olfactory Memory

A complex circuit involving all of these brain structures underlies olfactory memory processing (**Figure 3.1.3)**. In olfactory conditioning, input from olfactory receptors is projected via the antennal nerve to glomeruli in the antennal lobe, where they synapse with olfactory projection neurons (PNs). These cells project via the antennal-cerebral tract to the MB, where they provide presynaptic inputs to the MB, and continue to the lateral horn. Unconditioned stimulus (US) inputs to the mushroom body are mediated by dopaminergic signaling to D1-like dopamine receptors (DopR) in the γ lobes, where the initial association of the CS (odor) and US occurs (Qin et al., 2012). This information is conveyed amongst interconnected neurons within the γ, α'/β' and α/β lobes. The precise roles of the α/β, and α'/β' lobes are not completely understood. However, several studies demonstrate that the α/β lobes are essential for LTM (Pascual and Préat, 2001; Akalal et al., 2006; Yu et al., 2006). Other work indicates that the α'/β'

lobes are important during the first hour of memory consolidation, while 3h memory retrieval occurs through the α/β lobes (Krashes et al., 2007). Information is also conveyed via the MB to several extrinsic neurons, which form reciprocal connections with the MB that includes peptidergic, serotonergic, and gap junction signaling (Chen et al., 2012b; Lee et al., 2011; Pitman et al., 2011; Waddell et al., 2000; Wu et al., 2011). These extrinsic neurons are all required for different components of olfactory memory, suggesting that at least some degree of parallel processing underlies different forms of memory (STM, MTM, ARM, LTM). Recurrent activity between the α'/β' lobes and the APL and DPM neurons are required during intermediate stages of memory formation (Pitman et al., 2011; Wu et al., 2011), and serotonergic signaling from the DPM neurons are required for ARM (Lee et al., 2011). The DAL neurons, conversely, are required for LTM maintenance and retrieval, and form reciprocal connections with the MB that may occur preferentially in neurons that innervate the α/β lobes (Chen et al., 2012b). The R2/R4m neurons of the EB are also reciprocally connected with the MB (Zhang et al., 2013b). In the R2/R4m neurons NMDA receptors, presynaptic inputs from the MB, and synaptic transmission are all required for olfactory LTM, suggesting that MB to R2/R4m signaling is likely to be glutamatergic (Wu et al., 2007). R2/R4m neurons are both cholinergic and GABAerigc, though only the GABAergic neurons have been shown to input onto the MB (Zhang et al., 2013b).

Systems Consolidation in Drosophila

A number of studies in *Drosophila* indicate that over several days, associative olfactory memory is refined and consolidated, and this requires different subcomponents of the olfactory classical conditioning circuit over time. Based on this evidence, it has been concluded that in addition to molecular events that stabilize cellular change, mechanisms of systems consolidation occur to support LTM in flies (Dubnau and Chiang, 2013). Some of the strongest evidence for temporal phases of systems processing falls into two classes (summarized in **Figure 3.1.4**). The first is based on functional studies that inhibit synaptic transmission in particular subsets of neurons over different temporal windows at or after training. These studies identify neurons that are required only at particular times for LTM, or are specific to particular phases of memory. For instance, these studies show that synaptic transmission is required in the α′/β′ lobes during acquisition for STM, but is not required for retrieval (Krashes et al., 2007). Conversely, the α/β lobes are required during retrieval for LTM, but are not required during acquisition (Dubnau and Chiang, 2013). Synaptic transmission is required from the DAL neurons during a 12h window after training, and during retrieval, but not within the first 12h after training (Chen et al., 2012b).

The second class of evidence is based on studies in which the fly receives spaced training, and calcium imaging is used in particular lobes during re-presentaton of the paired odor (Davis, 2011). Here, I refer to these traces as 'retrieval traces'. These calcium imaging studies identify retrieval traces in the first hour after training in the α′/β′ lobes, the APL neurons, and the DPM neurons during early and middle-term stages (0-3h),

later traces in the α/β lobes, and very late traces lasting as long as 4 days in the γ lobes (Davis, 2011).

Is CREB required in the MB?

Ubiquitous expression of dCREB2-b, a dominant negative form of CREB at the time of training prevents LTM (Yin et al., 1994). While it has been traditionally assumed that CREB is required at the site of initial integration (the MB), a recent study has raised questions about the requirement for protein synthesis in the MB for LTM (Chen et al., 2012b). Expression of the ribosomal inhibitor RICIN, or expression of dCREB2-b in the MB does not affect 24h memory. Instead, this group finds that protein synthesis is required in the extrinsic DAL neurons. However, another group has suggested that the reason for this finding is a transgene dosage effect, and demonstrate both LTM impairment from dCREB2-b using another driver and induction system, and an enhancement effect that is mediated by MB dCREB2 (Hirano et al., 2013). While this effect may be due to partially to dosage, our results suggest that the MB contains two populations of neurons where dCREB2 may play opposing roles.

Figures and Legends

Figure 3.1.1

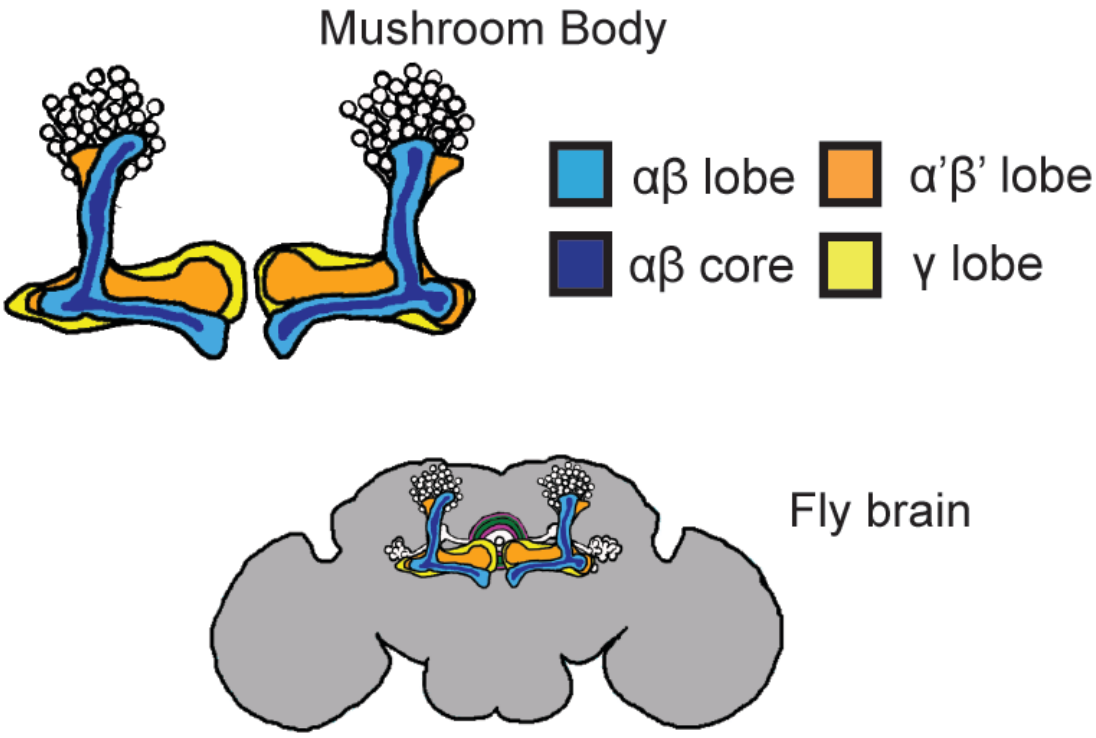

Figure 3.1.1: Cartoon depicting the structure of the mushroom body. A frontal view of the fly brain and the mushroom body is depicted. Cell bodies are shown in white, and different lobes (axonal processes) are shown in color

Figure 3.1.2

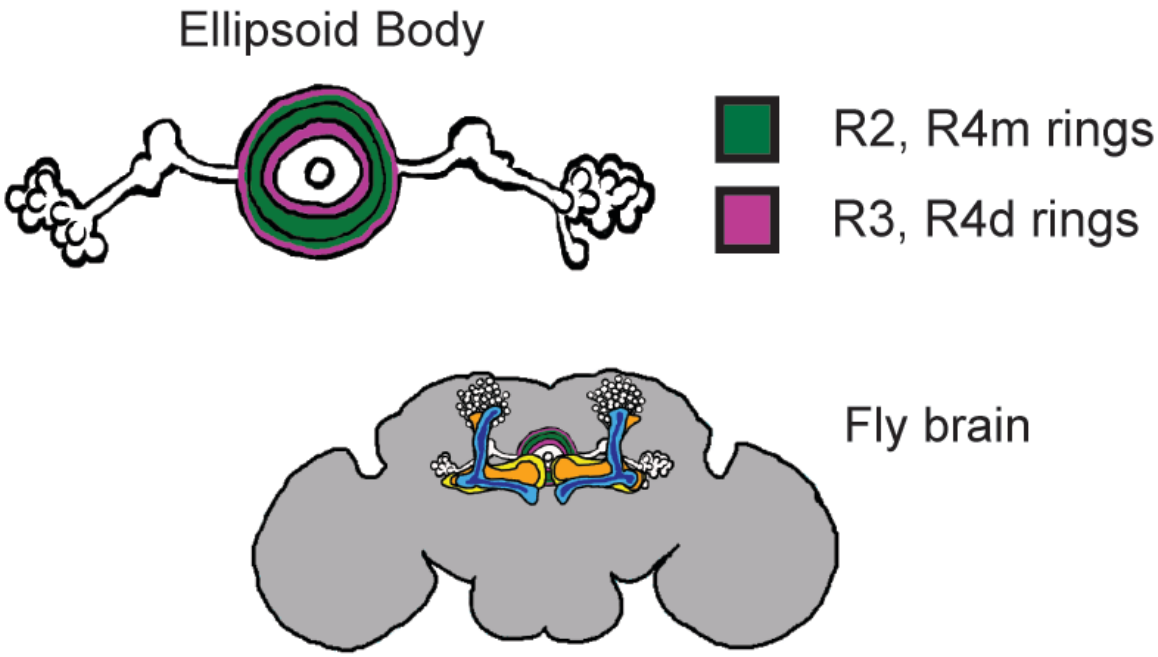

Figure 3.1.2: Cartoon depicting the structure of the ellipsoid body. A frontal view of the fly brain and the ellipsoid body is depicted**.** Ring structures corresponding to different GAL4 driver lines discussed in this chapter are shown in color.

Figure 3.1.3

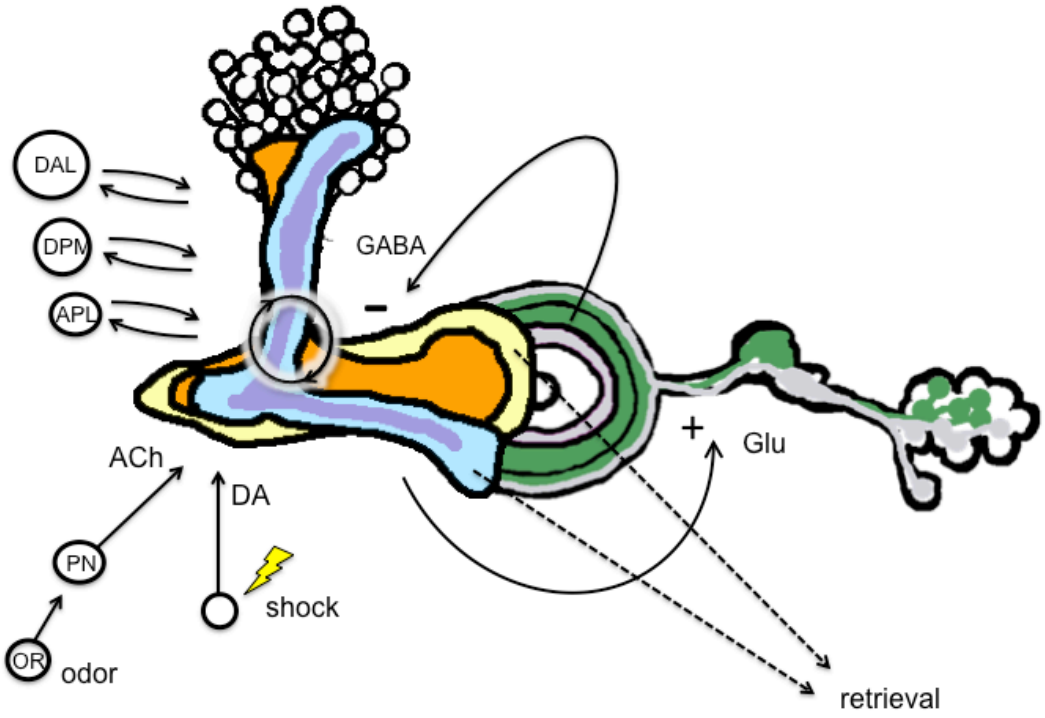

Figure 3.1.3: Neurocircuitry of olfactory classical conditioning memory. Odor is detected in olfactory receptor neurons in the antenna (OR), and transmitted to the mushroom via projection neurons (PN) in the glomeruli. Aversive information is conveyed to the mushroom body (MB) through dopaminergic projections, and the shock-odor association is integrated in the γ lobe (yellow). Neurons amongst MB lobes (αβ, blue), (α'β', orange) are interconnected, and to several extrinsic neurons: the dorsal anterior lateral (DAL) neurons, the dorsal paired medial (DPM) neurons, and the anterior paired lateral (APL) neurons. There are reciprocal connections between the MB and R2/R4m neurons in the EB (green).

Figure 3.1.4

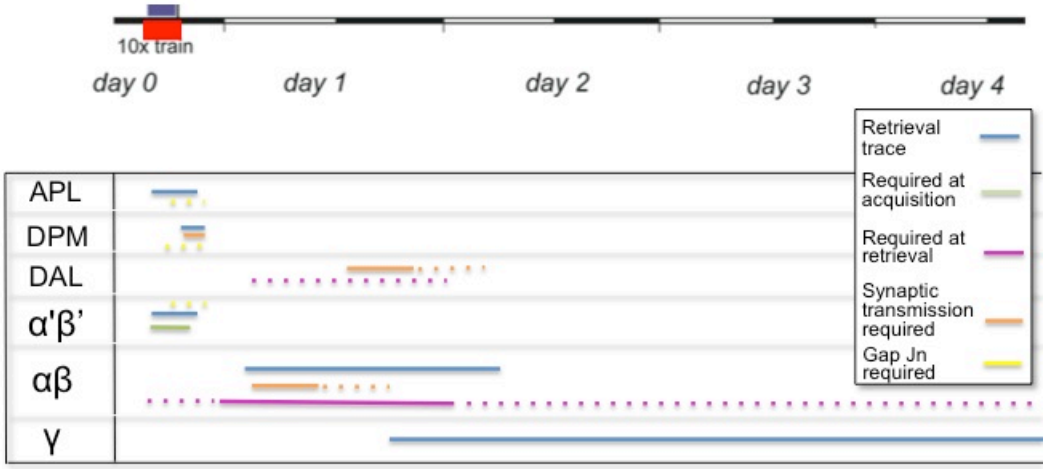
10x train
day 0
day 1
day 2
day 3
day 4
APL
DPM
DAL
α'β'
αβ
γ
Retrieval trace
Required at acquisition
Required at retrieval
Synaptic transmission required
Gap Jn required

Figure 3.1.4: Temporal phases of processing. Blue lines reflect time periods of calcium-imaging retrieval traces. Pink lines demonstrate requirements for synaptic transmission during retrieval (synaptic transmission is blocked during testing). Orange lines demonstrate a general requirement for synaptic transmission (synaptic transmission is not blocked during testing). Solid lines indicate demonstrated temporal windows, dotted lines indicate requirements where the temporal window is undetermined. These results are compiled and summarized from data presented and reviewed in: (Chen et al., 2012b; Davis, 2011; Dubnau and Chiang, 2013; Pitman et al., 2011; Wu et al., 2011).

3.2: Spatio-temporal *in vivo* recording of dCREB2 dynamics in *Drosophila* LTM processing

This section is a manuscript that has been submitted for review in its current form, except that references and figure headings have been adapted to the format for this dissertation.

Zhang J*, Tanenhaus AK*, Davis JC, Hanlon BM, and Yin JCP

* equal contributing co-authors

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 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 activity is suppressed immediately following LTM-specific training, and elevated during late windows. In addition, we observed heterogeneous responses of dCREB2 activity across different subsets of neurons in MB αβ 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.

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) lasting 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) and *de novo* protein synthesis (Chen et al., 2012b; Tully et al., 1994; Yin et al., 1994). CREB 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 believed to be required around the time of training (Alberini, 2009; Chen et al., 2012b; Yin et al., 1994). However, several studies in both mammals and *Aplysia* also suggest that the LTM-inducing training might produce biphasic effects on CREB activity, lasting for many hours(Bernabeu et al., 1997; 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, generating molecular information within a relatively limited 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., 2012b; Pascual and Préat, 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., 2012b; 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. We have developed a FLP recombinase-activated luciferase reporter for dCREB2 to monitor its transcriptional activity *in vivo*. 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 activity *in vivo* in brain regions of interest during the processing of LTM.

The aim of this study is to 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 activity in the EB R2/R4m neurons during the consolidation of LTM. We also uncover the complex and dynamic nature of dCREB2 activity in different subsets of MB neurons. Combined with previous studies, we propose that dCREB2 activity undergoes a series of dynamic modulations across different anatomical regions, and that this might be involved in the systems consolidation of LTM.

Results

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

We previously generated a luciferase-based dCREB2 reporter system that allows us to record dCREB2 activity in specific brain tissues in freely moving *Drosophila*. In the present study, we used the same tool to examine how LTM-specific training changes dCREB2 activity. To insure that the changes in dCREB2 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. A 1x FW training trial produces robust learning, but 1x BW training does not (**Figure 3.2.1 A, 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 (**Figure 3.2.1 A, middle panel**). The experimental design is summarized in **Figure 3.2.1 B**. Flies carrying both UAS-Flippase (UAS-FLP) and CRE-FRT-luciferase (CRE-F-luci) were crossed to different anatomically-specific GAL4 drivers. For each experiment, a group of flies expressing the dCREB2 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 the readout of dCREB2 activity. Since the only difference between the forward and backward training procedures is the shock-odor association, differences in dCREB2 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 some flies with 10x MFW and 10x MBW training protocol (**Figure 3.2.1 A, lower panel**), and then measured dCREB2 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 focus on two prominent brain regions that are important for LTM, the EB and the MB.

dCREB2 activity is increased in EB R2/R4m neurons for several days during LTM processing

Initially we focused on the EB, a brain region that is part of the central complex and forms a doughnut-like structure composed of classes of ring neurons (R1, R2, R3,

R4) (Renn et al., 1999). One 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 dCREB2 activity in EB is altered following LTM-inducing training, we expressed the dCREB2 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 dCREB2 reporter in the R2 and medial R4 (R2/R4m EB) neurons (Renn et al., 1999), the level of dCREB2 activity is significantly increased in 10x SFW-trained flies, lasting into the third day following training (**Figure 3.2.2 A**). To further confirm that the increases in dCREB2 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 dCREB2 activity is indicative of LTM, we expected to see a dependence upon 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 duration of increase in dCREB2 activity (compare **Figure 3.3.2 A** with **3.3.2 B**). We found no significant differences when flies were trained with a single training trial (**Figure 3.3.2 C**). This proportional relationship between memory duration and reporter persistence further supports the functional significance of the persistently elevated dCREB2 activity that we observed. 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 further confirm that

the elevation of dCREB2 activity is correlated with LTM, we examined the response following 10x massed training, which only produces ARM. We only observed a very subtle increase in dCREB2 activity following 10x massed training. This confirms that the persistent and dramatic increase in dCREB2 activity following 10x spaced training is specific to dCREB2-dependent LTM (**Figure 3.2.2 D**).

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 dCREB2 activity after training is not likely to be a general feature across the entire fly (**Figure 3.2.2 E**). This result suggests that our observed differences originate from certain tissues. When the reporter is expressed in eye cells, which are not thought to contribute to olfactory memory, there is no detectable difference between the memory group and the control group (**Figure 3.3.2 F**). 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 very close 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 does not lead to a long-lasting increase in dCREB2 activity (**Fig. 2D**). These data indicate that the LTM-associated persistent elevation of dCREB2 activity in the EB is restricted to R2/R4m neurons. A second R2/R4m-specific driver (c42) produces effects that are similar to those seen with c547 (**Figure 3.2.2 H**). This driver line previously was used to show the requirement for NMDA-Rs in LTM

consolidation (Wu et al., 2007). Taken together, we have identified a long-lasting increase in dCREB2 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 dCREB2 activity in R2/R4m neurons is a property of LTM processing.

dCREB2 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 γ, α'β' and αβ 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 MB in this process, we expected that dCREB2 activity would be elevated in MB following training. Surprisingly, the initial dCREB2 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 (**Figure 3.2.3 A**). However, we observed a trend towards an increase in dCREB2 activity beyond the first day (**Figure 3.2.3 A**). To examine whether this suppression in CREB 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 3.2.1**). c320-GAL4 expresses in αβ, 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 (**Figure 3.2.3 B**). However, the c739-GAL4 driver (which labels αβ lobe neurons), and the 1471-GAL4 (which labels the γ lobe), both showed dramatic decreases in dCREB2 activity immediately after the end of training. Notably, the suppression of dCREB2 activity based on c739 or 1471 is even stronger than that based on ok107, though they label fewer MB neurons (**Figure 3.2.3 A,C,D**). Intriguingly, dCREB2 activity during the third day or fourth day was significantly increased in the memory group, indicating that dCREB2 may still have functions to support LTM in the late windows (**Figure 3.2.3 C, D**). These results suggest that LTM-inducing training results in distinctive changes in dCREB2 activity across different MB neurons.

These heterogeneous changes in dCREB2 activity led us to examine more specific GAL4 drivers. Because multiple studies have suggested that the αβ lobe is important for LTM (Dubnau and Chiang, 2013; Hirano et al., 2013; Pascual and Préat, 2001), we tested several GAL4 drivers that can drive gene expression in this lobe, including c747, c772 and 17d. The MB αβ 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., 2012, 2013; Pavlopoulos et al., 2008). Interestingly, we found that 10x SFW training resulted in an elevation, but not suppression, of dCREB2 activity in c747-GAL4-driven MB neurons (**Figure 3.2.3 E**). A driver line that produces a similar pattern of expression (c772) showed a similar effect (**Figure 3.2.3 F**). Note that the fold-change in c772-expressing neurons is weaker (**Figure 3.2.3 F**), presumably because c772 also labels the γ lobe where dCREB2 activity is suppressed (**Figure 3.2.3 D** and **Table**

3.2.1). The observed changes in dCREB2 activity based on c747/c772 are very different from that based on c739. To further verify that the c739-responsive decrease and the c747- and c772-driven increases 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 (**Figure 3.2.3 G**). To gain more information, we then utilized another GAL4 line, 17d, which has restricted but strong GAL4 expression in the core region of $\alpha\beta$ lobe. We observed no differences between groups in the 17d-labled $\alpha\beta$ neurons following LTM-inducing training. Due to the lack of specific GAL4 lines, we were not able to locate the neurons where dCREB2 activity is decreased. However, our data suggest that the responses of dCREB2 activity are heterogeneous across different subsets of neurons in $\alpha\beta$ lobe.

Discussion

In this study we take advantage of a reporter fly (Tanenhaus et al., 2012) to explore dCREB2 activity *in vivo* during olfactory LTM formation. The ability to measure dCREB2 activity in restricted anatomical regions and over sustained periods of time after the end of training reveals the complex dynamics of dCREB2 activity during this process. The changes in reporter activity that we detect are very likely to be LTM-specific. This correlation is based on a number of key observations. Behaviorally, the sustained changes of dCREB2 activity in EB R2/R4m neurons are dependent upon forward pairing, their duration is a function of trial number, and are 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 in a driverless or anatomically irrelevant configuration (eg. the eye, R3/R4d), there is no detectable difference between the two activities. These results establish a strong correlation between LTM-specific behavioral parameters and relative changes in reporter activity. Our results also correlate anatomical regions important for LTM formation and reporter activity.

We detect a persistent increase of dCREB2 activity during LTM processing in a small subset of EB neurons that surprisingly can last for at least 3 days. 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 (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. 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 the excitability in the EB R2/R4m neurons is elevated for a number of days during the process of memory formation. The NMDA-Rs in these neurons are necessary for LTM consolidation, and our results support the possibility that these neurons (and dCREB2 activity within them) are involved in the consolidation/maintenance of late-LTM.

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. Our findings argue that the general MB GAL4 lines are not specific enough for dissecting the spatial distribution of dCREB2 activity during memory formation. This conclusion is based on the observation that an increase or decrease in dCREB2 activity does not segregate simply along identifiable anatomical lines. Since our measurement is the average 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. In principle, our approach could identify the LTM-specific regions of the brain if specific driver lines existed. However, in practice, at least with the lines that we tested, 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 ab drivers (c739, c747 and 17d). **Table 3.2.1** 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. The core region of the ab 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. We are confident that the difference in directionality that is detected is a function of LTM formation, since it is not seen when flies are trained with massed training. We suspect that quantitative differences in driver strength and the detailed sub-patterns of expression are responsible for the opposite directionality of reporter activity that we see. Further experimentation with more specific drivers should help to resolve these issues.

Nevertheless, the data we have gathered here strongly 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. Interestingly, we observed a small but significant increase in dCREB2 activity during the late phases of LTM. These changes are intriguing because they occur 3-4 days after the end of training, and in regions where the immediate changes are of opposite direction. Further investigation is required to strengthen these observations and test their importance.

Given the well-established positive roles of CREB in the formation of LTM, the correlation between LTM and decreases in dCREB2 activity are very surprising. What could be the role for suppression of dCREB2 activity in LTM? One possibility relies upon recent work demonstrating that baseline CREB activity can play a role in shaping systems-level memory encoding in mammals, likely by regulating excitability (Han et al., 2007; Yiu et al., 2014; Zhou et al., 2009). Notably, it has been suggested that the γ lobe is the gateway for US input to form the olfactory association (Qin et al., 2012), and that the core region of $\alpha\beta$ lobe functions as a gate for LTM formation (Huang et al.,

2012). Taken collectively, one intriguing hypothesis is that the suppression dCREB2 activity decreases the excitability in certain MB neurons, facilitating the gating of new LTM formation. The decreased neural excitability results in a higher threshold to form new memories in those neurons, thereby preventing previous memory processing from interfering with new memory formation. These observations and speculations imply the complex and dynamic nature of dCREB2 activity within LTM circuits, and provide the groundwork for future studies.

Regardless of the exact interpretation of our data, these results may in part explain the conflicting results of two recent studies that use different transgene induction tools to test the requirement for dCREB2 in the MB during memory formation (Chen et al., 2012b; Hirano et al., 2013). While it is possible that the gene dosage of an inhibiting transgene is responsible for the discrepant results, another possibility is that the bidirectional changes in dCREB2 activity predict 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.

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 and 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 within one set of neurons. These long-lasting changes and their anatomical complexity are consistent with an important role for dCREB2-responsive transcription in systems consolidation of memories. We believe that dCREB2 activity is needed acutely to mediate adaptive changes, but is also required chronically to sustain changes in neuronal state, such as excitability. This view predicts that each transition state in dCREB2 activity is likely to recruit the transcription factor, producing a complex picture of where and when acute dCREB2 activity is required during memory formation.

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., 2013b). The MB is also synaptically connected with DAL neurons where dCREB2 is required for LTM formation (Chen et al., 2012b). Taken collectively, all of these studies support a model of systems consolidation of *Drosophila* LTM (Dubnau and Chiang, 2013). 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.

Materials and Methods

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).

Olfactory aversive conditioning

Aversive Pavlovian olfactory learning and memory 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 10 time 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 a 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., 2013). 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).

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 in 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).

Statistical analysis

For statistical analysis of dCREB2 reporter data, we used a permutation test to test the null hypothesis of no forward training effect (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. Here, 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 learning and memory behavior, data were subjected to t-test (***: $p < 0.001$). Data were reported as mean ± SEM.

Figures, Tables and Legends

Figure 3.2.1

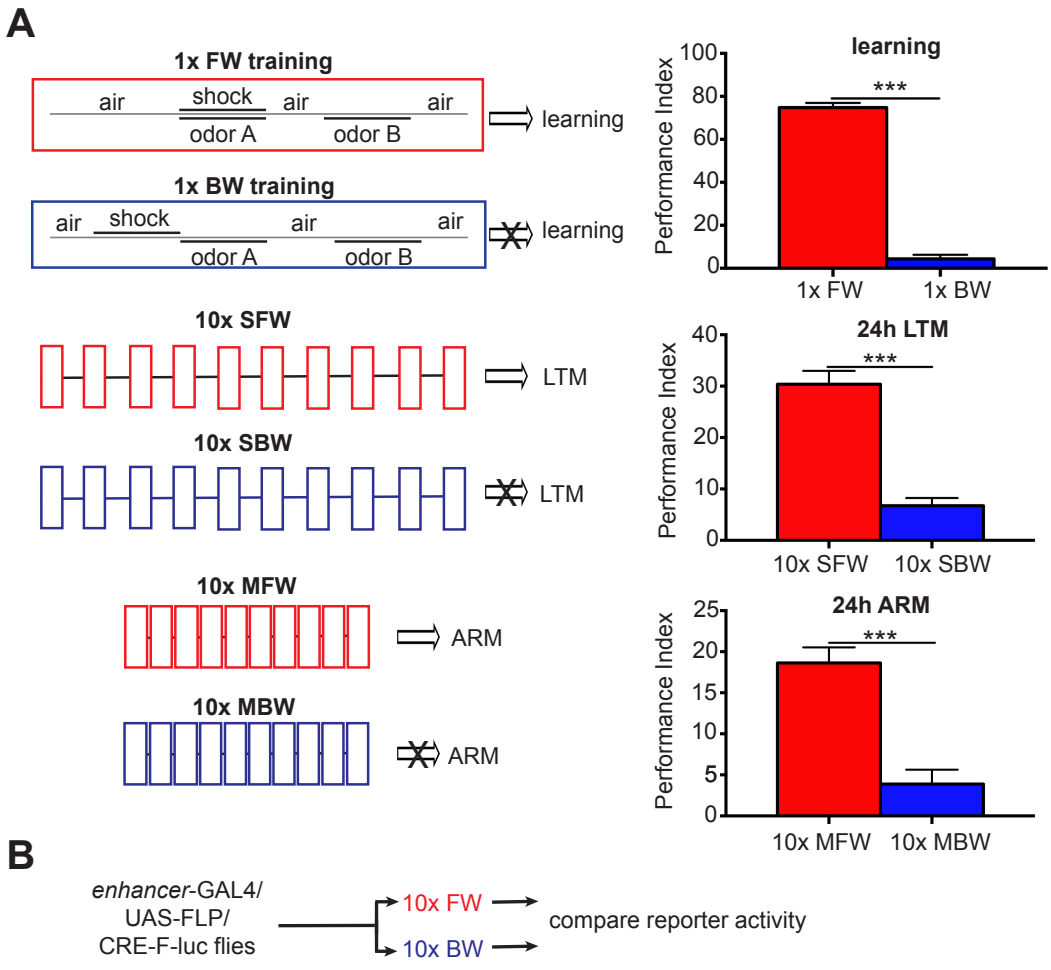

Figure 3.2.1. **Experimental design and behavioral training**

(A) Training protocols used to generate learning, 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 learning behavior, 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 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 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 3.2.2

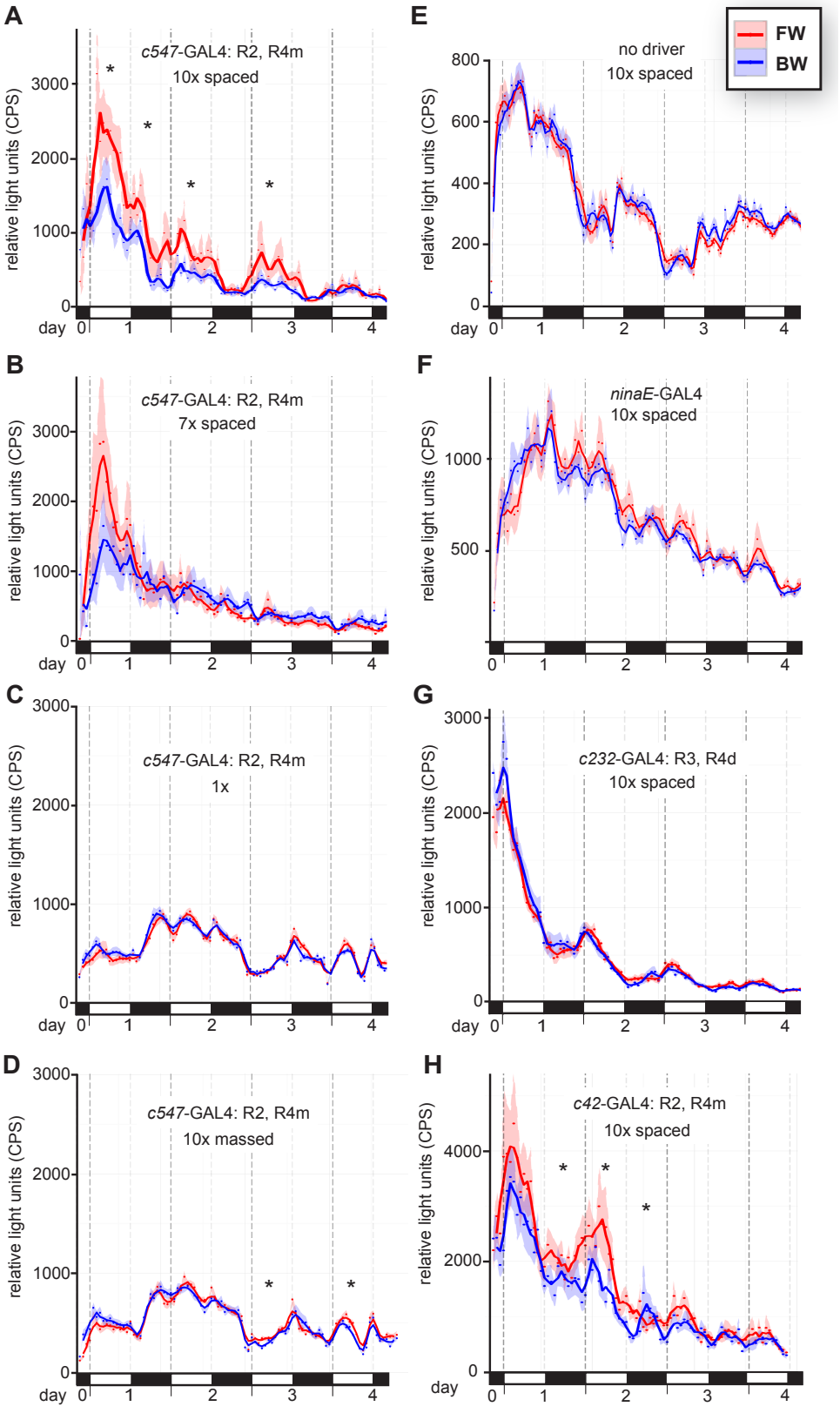
A
c547-GAL4: R2, R4m
10x spaced
B
c547-GAL4: R2, R4m
7x spaced
C
c547-GAL4: R2, R4m
1x
D
c547-GAL4: R2, R4m
10x massed
E
no driver
10x spaced
FW
BW
F
ninaE-GAL4
10x spaced
G
c232-GAL4: R3, R4d
10x spaced
H
c42-GAL4: R2, R4m
10x spaced
relative light units (CPS)
day

Figure 3.2.2. 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. (**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. **(C)** 1x FW training does not produce any significant increases in dCREB2 activity following training. **(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 interval between each training trial. **(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. **(F)** dCREB2 activity is not increased in eye cells following

LTM-inducing training. The expression of dCREB2 reporter is driven by ninaE (GMR) GAL4 driver (UAS-FLP/ninaE-GAL4; CRE-F-luc/+). **(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 close to but distinct from R2/R4m neurons using c232-GAL4. No persistent increase of dCREB2 activity was seen in the memory group. **(H)** Persistent elevation of dCREB2 activity in c42-GAL4-driven R2/R4m neurons after 10x SFW training . Asterisks indicate statistical significance for each day/night bin **(**$p<0.05$).

Figure 3.2.3

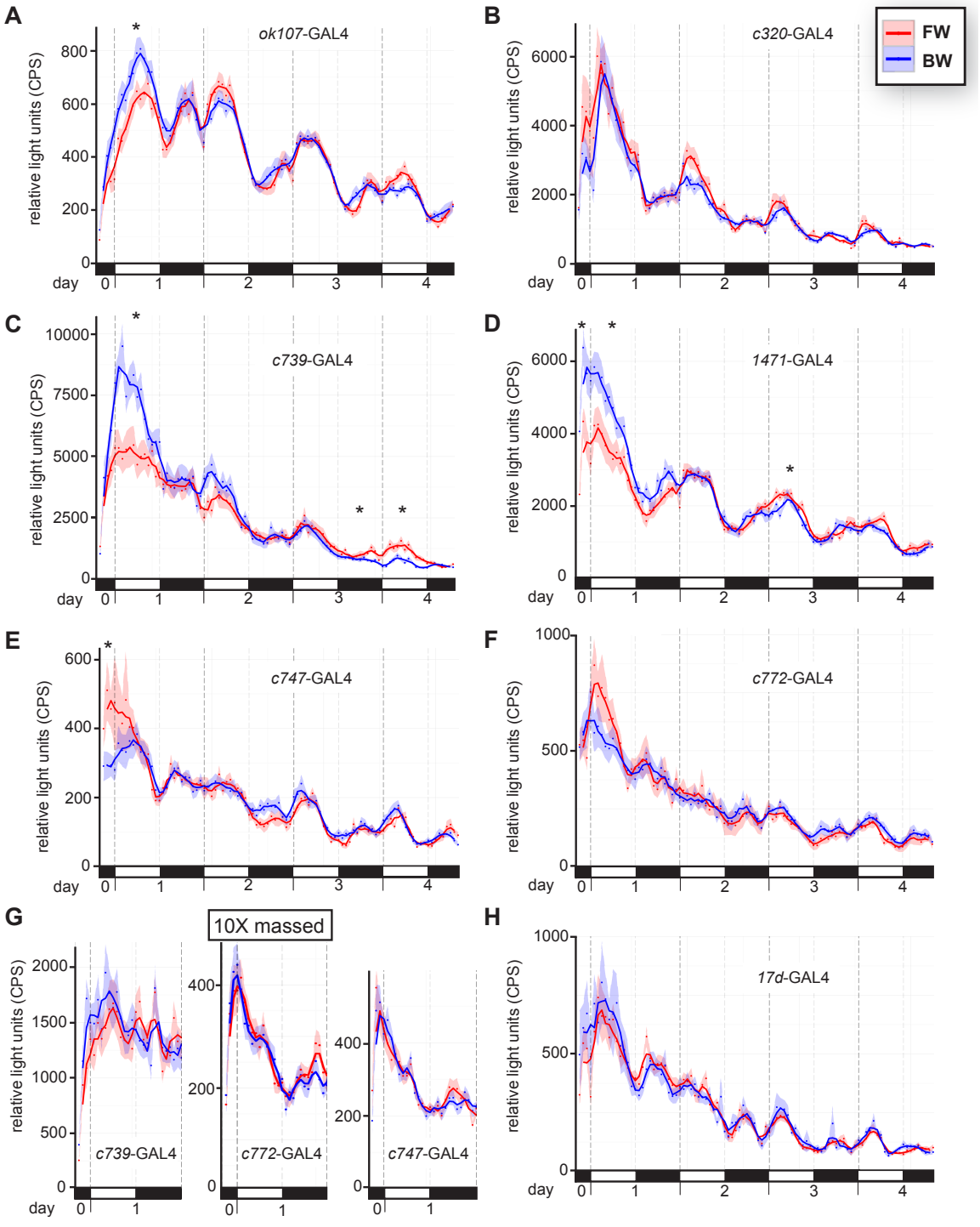
A
ok107-GAL4
B
c320-GAL4
FW
BW
C
c739-GAL4
D
1471-GAL4
E
c747-GAL4
F
c772-GAL4
G
10X massed
c739-GAL4
c772-GAL4
c747-GAL4
H
17d-GAL4
relative light units (CPS)
day

Figure 3.2.3. 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. **(B)** No significant change in dCREB2 activity in c320-labeled (preferentially in $\alpha'\beta'$ lobe, but also in $\alpha\beta$ and γ lobe) MB neurons following LTM-inducing training. **(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. **(E and F)** Increase of dCREB2 activity in subdivisions of MB $\alpha\beta$ lobe labeled by c747 and c772, respectively, following LTM-inducing training. **(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. **(H)** No significant changes in dCREB2 activity in 17d-labled (preferentially in the $\alpha\beta$ lobe core subdivision) following LTM-inducing training. Asterisks indicate statistical significance for each day/night bin **(**$p<0.05$).

Table 3.2.1

MB GAL4	γ main	γ dorsal	α'β' anterior	α'β' middle	α'β' posterior	αβ core	αβ surface	αβ posterior
ok107	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
c320		+++	+++++	++	+++++	++	++	+
c739						+++++	+++++	++++
1471	+++							
c747	+++	+++	++	++	++	++	+++++	+++++
c772	+++++	+++++	++	++	++	++++	+++++	+++++
17d						+++++	++	

Table 3.2.1. Expression pattern of MB GAL4 drivers used in Figure 3.2.3

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.

3.3: Wallenda regulates dCREB2 activity *in vivo*

Our data show that dCREB2 activity is downregulated during the first 12h of memory processing in cell populations under the control of a MB αβ-specific GAL4 driver (Figure 3.2.3C), suggesting, counterintuitavely, that suppression of dCREB2 activity in these cells may contribute to LTM processing. *In vitro* the human Wnd homolog, DLK down-regulates CREB activity by affecting both co-activators, CBP and TORC (Oetjen et al., 2006; Phu et al., 2011). Interestingly, when the MAPKKK Wallenda (Wnd) is overexpressed, or its upstream inhibitor, the ubiquitin ligase Highwire (Hiw), is blocked in αβ core neurons, single trial training is sufficient to generate LTM (Huang et al., 2012). This effect occurs within 12h following training, which is consistent with the timeline that we see for CREB suppression. Conversely, chronic loss of function of Hiw blocks 24hr memory (Huang et al., 2012), suggesting these cells are capable of facilitating or blocking LTM. Based on these data, it has been proposed that the αβ core region of the MB plays a gating role in LTM consolidation either permitting or blocking LTM formation. (Huang et al., 2012, 2013).

In combination with our data, we hypothesized that the enhancement effect of the Hiw-Wnd pathway on LTM formation may occur by suppressing dCREB2 activity in the MB αβ lobes. Therefore, we sought to test whether the Hiw-Wnd pathway modulates dCREB2 activity in *Drosophila in vivo*. Based on *in vitro* data, we predicted that overexpression of Wnd, or loss-of-function of Hiw should inhibit dCREB2 activity. To overexpress Wnd, we combined a UAS-driven Wnd transgene with the inducible pan-neuronal GAL4 driver, Elav-GeneSwitch. To block Hiw activity, we instead

overexpressed a dominant negative form of Hiw ($Hiw^{\Delta RING}$) which containes point mutations in the ubiquitin ligase RING domain (Wu, 2005). dCREB2 activity was measured in neurons by combining the CRE-F-luc reporter with an enhancer trap FLP line, 232B which expresses FLP pan-neuronally (Fore et al., 2011). Flies were raised on standard food, and then transferred to luminometer plates containing food fortified with luciferin and RU486, which activates the GeneSwitch system (Osterwalder et al., 2001). After a little over one day, consistent with the timecourse of feeding-dependent RU486 induction, dCREB2 activity was suppressed substantially in Elav-Geneswitch >UAS-Wnd flies, compared to genetic controls (UAS-Wnd) (**Fig 3.3.1 C**). Overexpression of $Hiw^{\Delta RING}$ produced a trend towards a suppression in dCREB2 activity with similar kinetics (**Fig 3.3.1 A**). The effect we see is much milder than what we see from Wnd overexpression. We attribute this to the fact that highwire is only endogneously expressed in a subset neurons, and therefore a dominant negative approach should not impact most of the cells that we are measuring. In the absence of drug induction, these effects were both attenuated (**Fig 3.3.1 B, D**).

These data suggest that in *Drosophila*, the Hiw-Wnd pathway regulates dCREB2 activity. Presumably, overexpression of Wnd or loss-of-function of Hiw in the MB $\alpha\beta$ core also suppresses dCREB2 activity. Taken together with our observation of dCREB2 suppression following LTM formation in c739-GAL4 expressing cells in the MB, it is possible that the LTM enhancement observed due to Hiw/Wnd pathway manipulation is due to targeted suppression of dCREB2 activity in these cells. If this is the case,

inhibition of dCREB2 activity in αβ core neurons during the first 12h following single trial training should facilitate LTM formation. Testing this possibility will require the ability to produce a precisely regulated spatio-temporal blockade of dCREB2 activity.

Figures and Legends

Figure 3.3.1

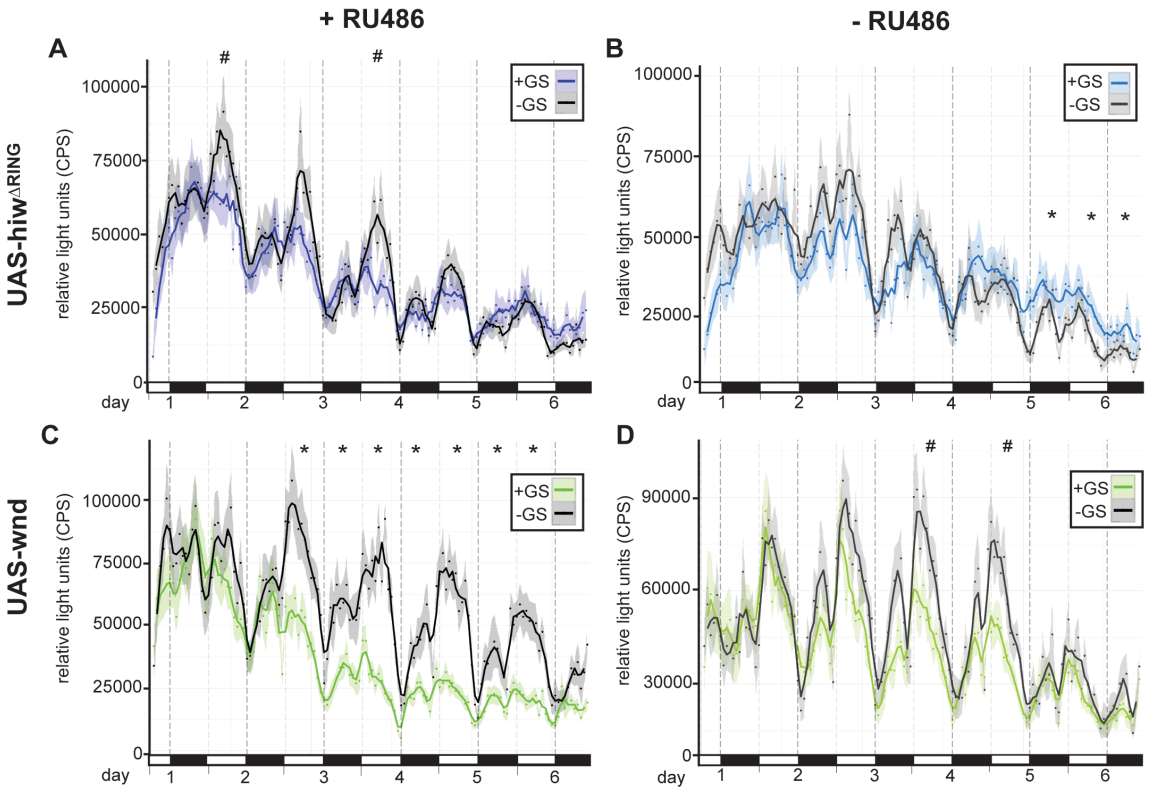

Figure 3.3.1. Wallenda suppresses dCREB2 actvity. Bioluminescence plotted over time in flies carrying the CRE-F-luc reporter activated by an enhancer-trap flippase line, 232B (pan-neuronal). (A,C) Flies carrying the UAS-Hiw$^{\Delta RING}$ with (GS+, blue) or without (GS-, black) the Elav-Geneswitch driver. Flies were placed on 500uM RU486-luciferin food at loading (A), or luciferin food with vehicle (DMSO) alone (B). (C,D) Flies carrying the UAS-wnd with (GS+, green) or without (GS-, black) the Elav-Geneswitch driver. Flies were placed on 500uM RU486-luciferin food at loading (C), or luciferin food with vehicle (DMSO) alone (D). Flies were entrained throughout development under 12:12 light dark conditions. Light conditions are indicated along the x-axis (black: night, white: day). Points indicate raw means for each data point, and ribbons indicate the SEM. Lines indicate a moving average of 3 data points. (* $p<0.05$, # $p<0.1$ for each day/night bin, permutation test)

3.4: General Discussion

What drives sustained changes in dCREB2 activity?

Through the course of memory processing, we observe persistent changes in dCREB2 activity that outlive the protein stability or activity of an acute dCREB2 response. Therefore, there must be other regulatory mechanisms that explain the persistent or periodic increases (EB, MB) and decreases (MB) in dCREB2 activity. One possibility is that intracellular feedback loops drive the maintenance of a high or low activity state for dCREB2. This may be the case in *Aplysia*, where a positive feedback loop drives chronic up-regulation of CREB activity (Liu et al., 2008). It is possible that the sustained changes in the EB may include some of these mechanisms. However, we do not favor this as a primary explanation for sustained changes here or in other complex organisms. We believe that a dCREB2-excitability-dCREB2 feedback loop would provide a reasonably simple explanation for the persistence of the chronic elevation (EB) or suppression (MB) of dCREB2 activity that we observe, as well as for late timing-dependent increases in the MB.

A hypothesized timeline for LTM processing in *Drosophila*

Immediate Phase (0-3h)

At the time of training, shock and odor coincidence is detected and encoded in the γ lobes, and conveyed throughout the MB, to the extrinsic MB neurons, and to the EB. Depending on the strength and nature of training, different subcomponents of the olfactory memory circuit will come online. Strong activity will push the recruitment of

those downstream circuits, and activate dCREB2 in some subset of these cells. This will lead to synaptic stabilization of connections that are ultimately required for LTM. A circuit composed of the α′/β′, APL, and DPM neurons will support the early maintenance and retrieval of the encoded information in the short term (0-3h) through parallel protein synthesis-independent mechanisms. Any transient dCREB2 activity that is recruited during this time is likely to occur at the time of training, and is therefore unlikely to show up in our assay.

Early Phase (1-24h)

One subpopulation of cells in the MB, likely comprised posterior α/β lobe neurons (for the purposes of this dissertation, I will call them A cells), interacts with the γ lobe. Strong activity will activate dCREB2, and recruit these cells to a more excitable state to help support the memory and facilitate retrieval in the intermediate term.

Meanwhile, strong training will activate R2/R4m neurons in the EB, and this will activate dCREB2 through NMDA-R-dependent calcium influx. Increased dCREB2 activity will lead to the expression of excitability-related proteins. This increased excitability of the R2/R4m EB neurons will produce a positive feedback loop that increases overall neuronal activity, thereby sustaining dCREB2 activity. Over time, homeostatic mechanisms will outcompete this positive feedback loop, and neuronal excitability will return to baseline. These neurons provide inhibitory inputs to the MB, likely to the subpopulation of neurons whose activity is suppressed.

Meanwhile, a second subpopulation of cells which includes cells in the γ and α/β lobe is inhibited (for the purposes of this dissertation, I will call them G cells), likely at least in part by GABAergic inputs from the EB. Neuronal activity from these cells gates consolidation, and they must be kept offline to permit LTM formation. This suppression of neuronal activity would down-regulate dCREB2 function (and vice versa), promoting LTM formation. There are several lines of evidence that are consistent with this possibility. The first is that there is a subpopulation of neurons in the α/β lobe that plays a gating role in LTM formation (Huang et al., 2012). Loss of function of highwire or gain of function of wallenda, which both suppress dCREB2 activity (**Section 3.2**), will permit LTM from a single training trial (Huang et al., 2012). The second is that NMDA-R overexpression in the R2/R4m neurons enhances LTM (Wu et al., 2007). While this effect could be explained if the olfactory memory itself is encoded in the EB, extensive studies implicating the MB in olfactory memory encoding make this unlikely (Akalal et al., 2006; Dubnau and Chiang, 2013). However, this could also be explained if NMDA-R overexpression lowers the threshold for the EB neurons to be activated, thereby inhibiting the G cells and permitting single trial LTM formation.

Late Phase (24h+)

Over time, as the memory becomes more consolidated, the subset of connections that ultimately encodes the memory trace is stabilized through the activity of a MB/DAL circuit (Chen et al., 2012b). This persists as EB-mediated inhibition is lifted.

This subset of cells is most likely to includes the γ lobes that acted as the original coincidence detectors, and encode the odor information, and a population of α/β neurons that support the memory trace and enable retrieval. This is consistent with data that show late retrieval traces in the γ lobe and in the α/β lobe (Davis, 2011), with a number of studies that suggest functional requirements for the α/β lobe in late LTM and retrieval (Dubnau and Chiang, 2013; Isabel et al., 2004). The reporter data described in this chapter show late dCREB2 activation using a γ lobe-specific driver (1471), an α/β lobe-specific driver (c739), and similar trends with a general MB driver (ok107), and a diverse driver that includes expression in most lobes (c320).

We believe that these late activations in dCREB2 activity represent site of final consolidation, and are best explained by long-lasting changes in neuronal excitability as follows: trace-specific cells are activated over the transition between the early and late phase of LTM processing. This process recruits dCREB2-dependent gene expression that includes proteins that are required for synaptic consolidation, and proteins that lead to up-regulation of intrinsic excitability in these cells. This excitability regulation is persistent enough that these neurons are preferentially reactivated during subsequent periods of time, which would resemble the phenomenon of 'replay'. This process would facilitate the continued reactivation of the dCREB2-excitability loop, and thereby the maintenance of synaptically-encoded memory circuits. The late periods that we observe where dCREB2 activity is activated appear to themselves be under circadian control, and tend to occur during daytime periods that coincide with endogenous sleep-correlated daytime peaks in dCREB2 activity. This is emphasized in **Figure 3.4.2**, where relative

differences between FW and BW groups are plotted, and is consistent with the idea that dCREB2 activity in general is organized by circadian mechanisms. This phenomenon that we observe is strikingly reminiscent of oscillations in cAMP-MAPK activity in the hippocampus, where MAPK activity is required during sleep-correlated daytime peaks for memory persistence (Eckel-Mahan et al., 2008).

Future Directions

This model is based on correlative observations, and is currently only speculative, but leads to several testable functional predictions:

1. Inhibiting dCREB2 activity in the MB α/β and γ lobes during late daytime windows should interfere with late LTM
2. Inhibiting dCREB2 activity in the R2/R4m EB neurons should affect late LTM
3. Specifically-targeted dCREB2 inhibition of G neurons should facilitate LTM
4. Specifically-targeted dCREB2 inhibition of A neurons should interfere with LTM

Figures and Legends

Figure 3.4.1

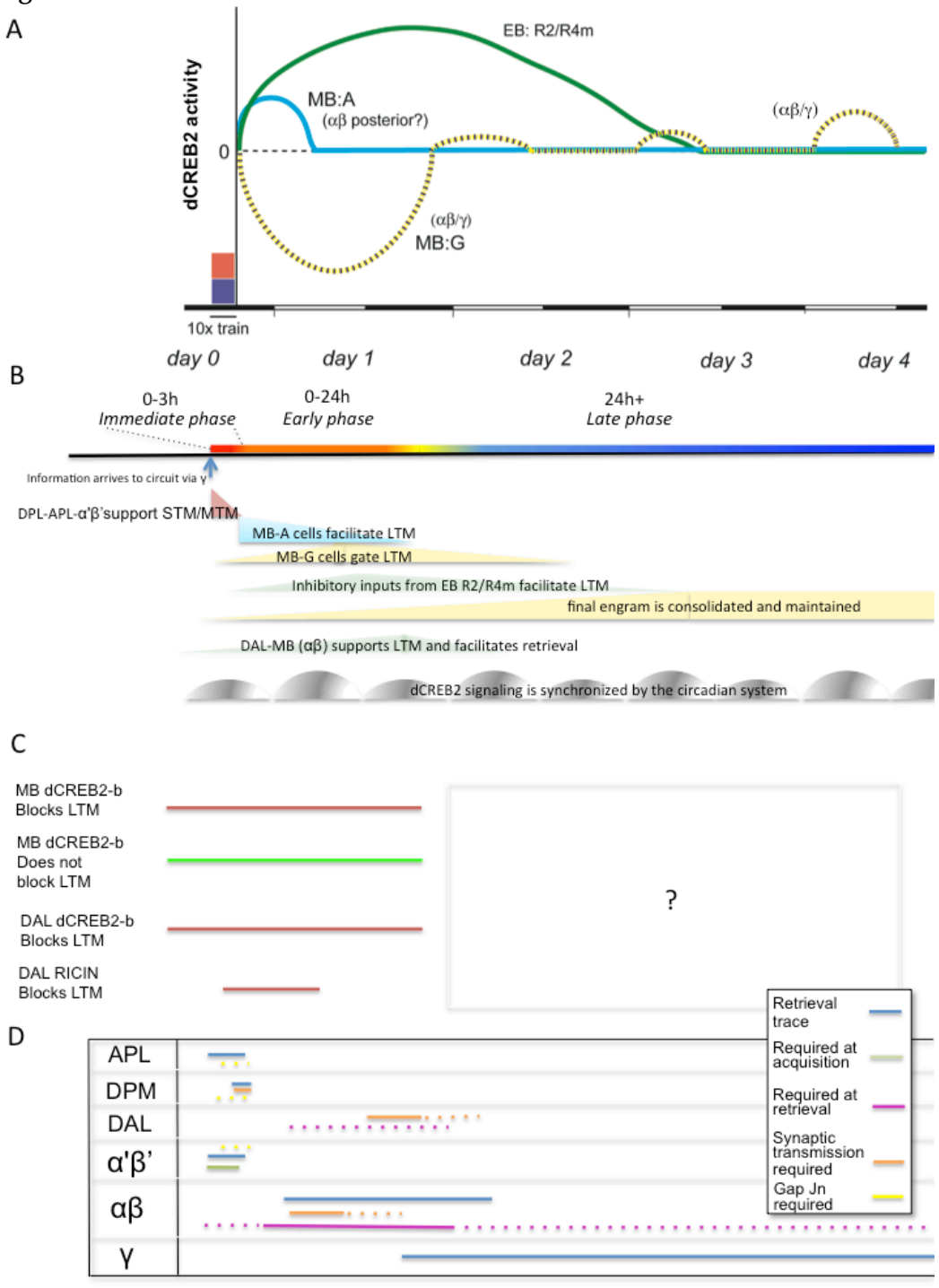

Figure 3.4.1: Hypothesized timeline for LTM processing in Drosophila. A) Summary of our observations of dCREB2 activity expressed as the relative difference in the FW group. Two classes of MB neurons that exhibit excitations (MB:A) or inhibitions (MB:G) in dCREB2 activity during the first day, and late recurring elevations in subsequent days. **B)** Summary of a hypothesized model for systems consolidation over time. **C)** Summary of dCREB2 functional data in the MB and DAL neurons (Chen et al., 2012b; Hirano et al., 2013). Lines indicate time during which dCREB2 is blocked. In both cases, 24h LTM was measured. D) Same data as described above (3.1.4), summarizing data from: (Chen et al., 2012b; Davis, 2011; Dubnau and Chiang, 2013; Pitman et al., 2011; Wu et al., 2011).

Figure 3.4.2

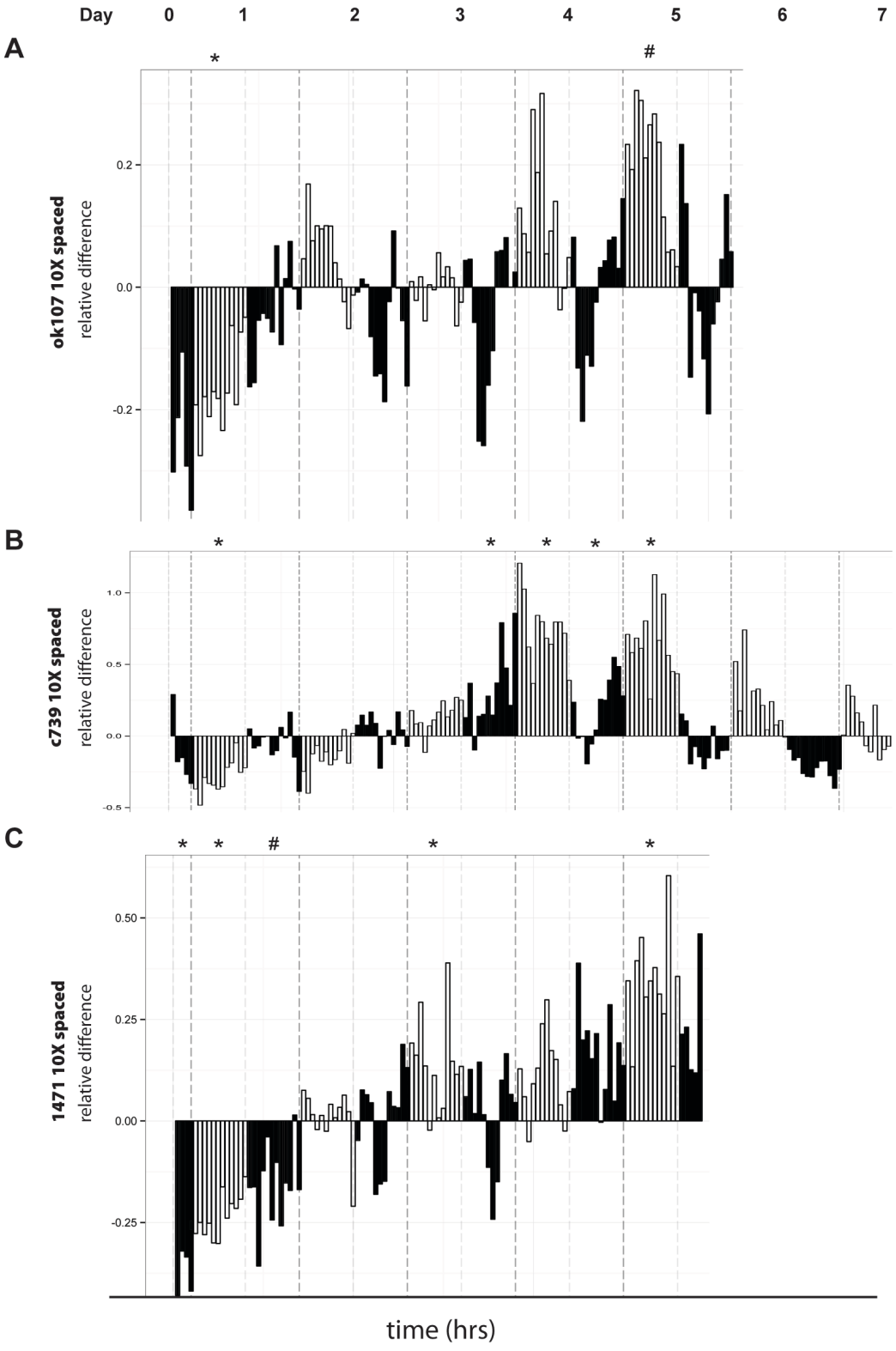
Day 0 1 2 3 4 5 6 7
A
B
C
ok107 10X spaced
relative difference
c739 10X spaced
relative difference
1471 10X spaced
relative difference
time (hrs)

Figure 3.4.2: Late forward-pairing dependent increases in CREB activity occur preferentially during daytime periods. The same data as previously shown (2.1 Fig 3A, C, D), except that additional recorded days are analyzed, and the data are expressed for each hourly time point as the relative difference between the means of the FW and BW groups [(FW-BW)/BW]. Data for daytime periods are plotted with white bars, and for nighttime periods are plotted with black bars. * Indicates statistical significance ($p<0.05$),

Acknowledgements: Chapter 3

3.2 We thank Sam Moen for luminometer repair, and Hong Zhou for technical assistance.

3.3 We thank C. Collins for providing UAS-wiw$^{\Delta RING}$ and UAS-wnd stocks, and K. Reed for assistance with sample collection and fly work.

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Chapter 4

Characterizing the phenomenology of memory enhancement

Introduction

In the previous two chapters, I show data characterizing the spatio-temporal pattern of dCREB2 activity both at baseline and during memory consolidation. In this chapter, I begin to explore the phenomenology of memory enhancement, with the goal of ultimately understanding the rate limiting steps for memory formation.

4.1 Background

CREB and Memory Enhancement

One of the most remarkable features of memory is that it can be enhanced. Throughout this chapter, I define enhancement specifically as the ability to form memories from normally sub-threshold stimuli. This can often be biologically beneficial - for instance, the ability to remember very important events from a single encounter can be critical for survival. This can also be biologically detrimental, and inappropriate plasticity can contribute to neurological and neuropsychiatric dysfunction. From a therapeutic perspective, gain-of-function effects in memory formation are particularly promising. For instance, while understanding different etiologies of memory disorders can help drive specific treatments, targeted techniques to improve memory could be a broad strategy for treating a wide variety of memory disorders or cognitive deficits. However, compared to loss of function memory phenotypes, gain-of-function phenotypes are significantly understudied.

Perhaps the most interesting features of CREB activity are revealed in studies of memory enhancement. These studies that show that gain-of-function in CREB activity

can, in particular contexts, lower the threshold for memory formation. In *Drosophila*, the global expression of a heat shock-inducible dCREB2 activator isoform (*hs*-dCREB2a) lowers the behavioral threshold for long-term memory formation, both for olfactory classical conditioning, and for courtship conditioning (Tubon et al., 2013; Yin et al., 1995). Likewise, food deprivation lowers the threshold for long-term memory formation by increasing dCREB2 activity via the co-activator TORC (Hirano et al., 2013). CREB-mediated memory enhancement is also conserved in mammals. Improved performance in a series of memory tasks has been reported in $CREB^{DIEDML}$ and $CREB^{Y134F}$ mice, which both express activated CREB forms (Suzuki et al., 2011). In hippocampus, viral expression of the $CREB^{Y134F}$ form enhances contextual fear conditioning (Restivo et al., 2009). Similarly, in amygdala viral-mediated CREB overexpression in a random subset of neurons enhances memory formation for fear conditioning (Han et al., 2007, 2008; Josselyn et al., 2001b; Yiu et al., 2014). Importantly, these amygdala studies show that CREB overexpression does not lead to the recruitment of more cells to support the memory trace, but rather biases those particular cells toward participation in memory encoding. Together, this suggests that in addition to shaping the allocation of the memory, it also lowers the threshold for consolidation at an intracellular level.

CREB is one of only a handful of molecules identified whose global (brain-wide) manipulation can lead to memory enhancement. Furthermore, a number of these molecules (e.g. notch, ecdysone, period) and behavioral treatments (e.g. starvation) that can cause memory enhancement regulate CREB (Hirano et al., 2013; Ishimoto et al., 2009; Sakai et al., 2004; Zhang et al., 2013a). These observations position CREB as a molecular

bottleneck in memory formation. However, at least in *Drosophila,* dCREB2-mediated enhancement of memory formation does not occur under all conditions (Perazzona et al., 2004; Tubon et al., 2013; Yin et al., 1995). For instance, in olfactory classical conditioning memory enhancement produced by induction of *hs*-dCREB2-a depends on time-of-day: global induction of dCREB2-activator does not enhance memory formation at a mid-day time point (ZT=6), but does enhance memory formation at a late-day time point (ZT=11) (Tubon et al., 2013). Suggestively, ZT=6 corresponds to a relative peak in endogenous dCREB2 activity over the circadian cycle, while ZT=11 corresponds to a relative trough in dCREB2 activity (**see Chapter 2**). Similarly, dCREB2-activator induction more consistently enhances memory for courtship conditioning than for olfactory classical conditioning, which have very different experimental conditions and underlying mechanisms. Finally, pre-training crowding will block dCREB2-mediated enhancement, without affecting learning (personal communication, E. Friedman). Interestingly, one major difference is that flies are typically kept in groups of 100 before and during olfactory training, while for courtship they are typically isolated or kept at very low density and trained individually. All of this suggests that there are other constraints that determine whether or not dCREB2 activity can act as a rate-limiting step for memory formation. Over many years, we have noticed that conditions that are typically more favorable to memory formation in general tend to be less favorable to enhancement, but have only a partial understanding of what these conditions are (Tubon et al., 2013).

Taken together, it appears that there are certain cellular or physiological states that are permissive to memory formation, and certain states that are less permissive to memory formation. While dCREB2 gain-of-function can permit sub-threshold memory formation, perhaps by switching cells into a more permissive state, this can only occur during otherwise non-permissive states. It is not well understood what molecular or physiological constraints determine whether enhancement can occur or not, or why they do. However, if dCREB2 activity is a key feature in one of several interrelated mechanisms that are capable of permitting molecular consolidation, other states should modulate the ability of dCREB2 to enhance memory formation. For instance, one prediction is that when neuronal excitability is low, (due either to dCREB2-dependent or dCREB2-independent mechanisms), increasing dCREB2 activity should permit sub-threshold memory formation. When neuronal excitability is otherwise high, increasing CREB activity should not permit sub-threshold memory formation. We hope that better characterizing the phenomenon of enhancement will lend insight into the nature of enhancement itself, and may help inform therapeutic strategies.

In order to better understand the factors that regulate memory enhancement experimentally, we chose to study enhancement in the courtship conditioning paradigm, where dCREB2 activation more consistently enhances LTM.

Courtship Conditioning

Courtship conditioning is an associative learning and memory paradigm, where male flies are conditioned to suppress their courtship behavior towards female flies.

Male flies exhibit a stereotyped set of courtship behaviors towards female flies that includes a sequential series of behaviors. Typically this begins with the male fly orienting towards the female fly, tapping her with his forelegs, producing a wing song, licking her, attempting copulation, and if she permits, copulating with the female fly (Sokolowski, 2001). Female flies that have previously mated will typically not mate again. They carry a number of chemical cues that are transferred from their previous mating partner, and display rejection behaviors towards subsequent male flies (Griffith and Ejima, 2009; Siegel and Hall, 1979). Pairing a virgin male fly with a mated female fly will lead to the suppression of his courtship behavior towards her, and to subsequent virgin and non-virgin flies (Siegel and Hall, 1979). This suppression behavior can be harnessed experimentally, and courtship suppression memory has been commonly used as a learning and memory assay (Griffith and Ejima, 2009). Typically, a virgin male is paired with a pre-mated female for a training period, during which the male attempts to copulate with the female but is rejected. Subsequently, the same male is presented with a new female (typically a virgin female), and the amount of time the male fly spends in courtship behaviors is quantified. Less time spent in courtship behavior is indicative of greater courtship suppression memory.

As opposed to olfactory classical conditioning where the unconditioned stimulus (shock) and conditioned stimulus (odor) are precisely defined, courtship conditioning can rely on multimodal sensory input. Several chemical cues are sufficient to act as an unconditioned stimulus, and visual and olfactory cues are sufficient but not necessary to act as a conditioned stimulus (Ejima et al., 2005, 2007; Joiner and Griffith, 1997, 2000).

Consistent with this, different brain areas that receive different types of sensory input will support courtship conditioning memory under different conditions (Joiner and Griffith, 1997, 1999, 2000). Like other associative paradigms, the duration of memory is modulated by the duration of training, and very long pairing periods can produce LTM lasting as long as 7-10 days (McBride et al., 1999; Siegel and Hall, 1979).

4.2: Crowding stress, dCREB2-activator induction, and RU486 feeding enhance courtship suppression memory

Tanenhaus AK, Wong J, and Yin JCP

Introduction

cAMP Response Element Binding protein (CREB) is established as a conserved mediator of long-term memory (LTM) formation. In both mammals and in *Drosophila*, activation of dCREB2 is capable of enhancing memory formation (Josselyn et al., 2001; Yin et al., 1995). In *Drosophila*, gain-of-function in dCREB2 activity can be produced by the overexpression of an activator dCREB2 isoform (dCREB2-a). The transgenic construct is based on an endogenous dCREB2 variant whose protein product is generated from an internal translation initiation site. This product is a transcriptional activator, and when activated under the control of a heat shock promoter leads to an up-regulation of CRE-mediated gene expression over a 90m window (Tubon et al., 2013). However, in *Drosophila* this global overexpression of dCREB2-a enhances olfactory classical conditioning memory intermittently, which has generated some controversy over its ability to enhance at all (Perazzona et al., 2004). Recently, we have reaffirmed the enhancement effect, and in doing so identified several interesting features of dCREB2-a-mediated enhancement (Tubon et al., 2013). Notably, this enhancement effect is particular to specific times of day, where activator induction and training at times that coincide with high endogenous dCREB2 activity are poor times for memory enhancement. Moreover, enhancement tends to occur when baseline memory formation is low. This has led us to the view that dCREB2 plays a gatekeeping role in molecular

consolidation, and can permit sub-threshold memory formation only during some otherwise non-permissive state. However, we have still not identified what these states are, and have only an incomplete understanding of what enhancement represents biologically. In a courtship conditioning paradigm, induction of dCREB2a under the control of a heat shock promoter (*hs*-dCREB2a) more predictably produces memory enhancement. Therefore, we sought to take advantage of this feature to begin to explore which factors modulate memory enhancement.

Methods

Fly Lines

All flies stocks were raised on standard cornmeal-molasses food. Males for courtship conditioning were collected as virgins, and housed at 22°C and 70-80% relative humidity in groups of 10 male flies. *hs*-dCREB2-activator flies (807) (Tubon et al., 2013), and CRE-dEGFP flies were generated in the lab using standard methods (BestGene). Fly lines were generously provided by M Nitabach (UAS-NaChBac, UAS-dORKΔC, and UAS-dORKΔNC, Elav-GeneSwitch), and Canton S flies were obtained from P Shaw.

Courtship Conditioning

Training conditions were similar as reported previously (Sakai et al., 2004; Tubon et al., 2013). The day before training, mated female flies were prepared in groups by combining wild type (Canton S) virgin female flies (3-5 days old) with an equal number of male flies (5-14 days old). We observed that when virgin males were used for premating, these conditions led to copulation in close to 100% of the female flies. On the day of training, virgin male flies (3-5 days old) were paired with a mated female flies (3-5 days old) in an acrylic training chamber (5mm deep x 15mm in diameter) containing 1ml agar-sucrose food (1% agar, 5% sucrose). For each experimental group, a naïve condition was run in parallel, where flies were transferred to training chambers alone for the duration of training. Training sessions were recorded, and flies that copulated during the training period were excluded from analysis. Following training, flies were

transferred into individual vials containing standard food. Long-term memory was tested 3 days following training by presenting males with a frozen virgin female fly (wild type, Canton S) in a testing chamber (3x15mm) containing damp filter paper. Testing sessions (10m) were recorded using a Logitech Webcam Pro 9000. A courtship index (CI) for each fly was determined by a blind observer, as quantified by the percentage of time the fly spent in courtship behaviors. Except for during heat shock, flies were maintained at all times under 12:12 light:dark conditions at 22°C and 70-80% relative humidity at all times. Because courtship scores are not normally distributed, a Mann-Whitney U test was used to test for statistically significant courtship suppression for each trained group compared to the matched naïve group ($p<0.05$).

Crowding

For crowding experiments, virgin male flies were maintained in groups of 10 until 4 days old. Three hours before the beginning of training, three groups of 10 flies were combined into a single food vial, and cotton was positioned to allow 5cm (LoD), 1.5cm (MeD), or 0.5cm (HiD) of space between the cotton and the food.

Heat Shock

To induce *hs*-dCREB2a expression, housing vials containing groups of male flies were transferred to a 37°C air incubator for 30m. Following heat shock, flies were allowed 5m to recover at 22°C before the beginning of training.

Drug Feeding

RU486, or mifepristone (Tocris Bioscience) was dissolved in DMSO, and added to molten agar-sucrose food cooled to 50°C (≤2.5% final concentration of DMSO). In all drug experiments, control groups (0ug/ml) were transferred to food containing an equivalent amount of vehicle (DMSO). Flies were transferred to drug food vials 24h prior to training, and kept on drug food until the onset of training.

Results

We have noticed previously that housing density prior to training seemed to be a factor in determining enhancement. Therefore we sought to first characterize the effect of crowding alone on memory formation (**Figure 4.2.1**). We chose as our genetic control line a transgenic reporter fly (CRE-dEGFP) that was generated in the same genetic background as our other transgenic lines. Virgin male flies were kept under low density conditions until three hours of training, and then placed for three hours under conditions of low crowding stress (LoD) moderate crowding stress (MeD), or high crowding stress (HiD) before 5h training. Under normal conditions, and in our hands, 5h of continuous training is not sufficient to produce LTM, and no significant courtship suppression is observed 3-days or 5-days after training (Ishimoto et al., 2009; Sakai et al., 2004, 2012; Tubon et al., 2013). Interestingly, following moderate crowding stress, 5h training was sufficient to produce 3-day courtship suppression memory, but not following low crowding stress or high crowding stress (**Figure 4.2.1)** This suggests that within a particular window, crowding can be a positive regulator of memory formation.

CREB is a known regulator of synaptic plasticity and intrinsic excitability in mammals and in flies (Benito and Barco, 2010; Ghezzi et al., 2010; Wang et al., 2009). There are several mechanisms through which CREB gain-of-function might enhance memory formation. One possibility is that CREB reduces the threshold for behavioral memory by bypassing the requirement for *de novo* gene expression that supports synaptic plasticity. Elegant studies demonstrating synaptic tagging suggest that this is a viable explanation (Frey and Morris, 1997). A second possibility is that gain-of-function in CREB activity also leads to a state in which long-term memory is able to form by increasing excitability. Because ubiquitous activation of dCREB2 is sufficient to enhance courtship memory, as a first pass we therefore asked whether increasing neuronal excitability ubiquitously is itself sufficient to enhance courtship memory. In flies, expression of a bacterial voltage-gated sodium channel (NaChBac) causes neuronal hyperexcitability by increasing action potential amplitude and duration (Hodge, 2009; Nitabach et al., 2006). Because pan-neuronal overexpression of UAS-NaChBac is lethal during development (Nitabach et al., 2006, our observations), we chose to express NaChBac pan-neuronally under the control of the inducible GeneSwitch system (Osterwalder et al., 2001). GeneSwitch is a chimeric GAL4 variant that is activated in the presence of the progesterone inhibitor RU486, allowing it to bind and activate UAS-dependent transgene expression. In larvae and adult flies, *elav*-GeneSwitch produces pan-neuronal expression of GeneSwitch, which is activated in a dose-dependent manner by RU486 feeding (Osterwalder et al., 2001). Doubly heterozygous *elav*-

GeneSwitch/UAS-NaChBac (*elav*-GS/NaChBac) flies were fed pre-fed with food containing varying concentrations of RU486 (0, 5, 50, 200μg/ml) (**Figure 4.2.2**). For any of these concentrations, beyond a mild hyperactivity we did not observe any locomotor or courtship defects following 24h or 48h drug feeding. To test for an enhancement effect of UAS-NaChBac induction, flies were pre-fed for 24h on drug food, and then transferred to standard food at the onset of 4h training. Interestingly, pre-feeding with either 5μg/ml or 200μg/ml, but not 50μg/ml RU486 resulted in significant 3-day courtship memory, suggesting that under some conditions increasing excitability may facilitate courtship memory.

Based on this result, we hypothesized that excitability is one of the key regulators of a permissive or non-permissive state for dCREB2-mediated memory enhancement. Therefore, we predicted that decreasing excitability might lower the threshold for dCREB2-mediated memory enhancement. Therefore, we sought to characterize the behavioral threshold for dCREB2-activator mediated enhancement of courtship memory. Long-term courtship memory has been reported following either 7h of continuous training, or 3 spaced 1hr training sessions (McBride et al., 1999; Sakai et al., 2004), while 5h of training is generally used to test for memory enhancement (Ishimoto et al., 2009; Sakai et al., 2004, 2012) In order to identify the optimal training time for memory enhancement, we asked whether dCREB2-activator induction produces LTM under several sub-threshold conditions: 2hr, 3hr, and, 5hr training (**Figure 4.2.3**). To induce activator expression, *hs*-dCREB2-a virgin males received a 30m heat shock treatment immediately prior to training, while an uninduced control group received

handling alone. When activator was induced prior to 2h training, no differences were observed between heat shock and no heat shock groups (**Figure 4.2.3 A**). When induced prior to 3h training, we observed a trend towards courtship suppression that did not reach statistical significance (**Figure 4.2.3 B**). When *hs*-dCREB2a was induced prior to 5h training, 3d courtship memory was significantly enhanced (**Figure 4.2.3 C**).

Therefore, to test the prediction that reducing baseline excitability might facilitate dCREB2 enhancement, we chose a 3h training protocol, just below the threshold for dCREB2 enhancement, and asked whether reducing neuronal excitability would permit dCREB2-activator enhancement of memory formation. To decrease excitability, we overexpressed a transgenic *Drosophila* open rectifier potassium channel dORKΔC, which acts as a leak channel (Nitabach et al., 2002), decreasing neuronal excitability by mildly suppressing resting membrane potential (Park and Griffith, 2006). As a control, we overexpressed a nearly identical transgene carrying a point mutation that renders the channel impermeable to potassium, dORKΔNC (Nitabach et al., 2002). Pan-neuronal expression of dORKΔC is also lethal during development (Nitabach et al., 2002, our observation), so we again expressed the dORKΔC or dORKΔNC transgenes under the control of *elav*-GeneSwitch. For each experiment, we generated flies expressing *hs*-dCREB2-a homozygously, a single copy of dORK, and a single copy of *elav*-GeneSwitch: *hs*-dCREB2-a; *elav*-GeneSwitch^{GAL4} / UAS-dORKΔC (*hs*-dCREB2-a-GS-ΔC) and dCREB2-a; *elav*-GeneSwitch/UAS-dORKΔNC (*hs*-dCREB2-a -GS-ΔNC). In *hs*-dCREB2-a-GS-ΔC flies, low level (5μg/ml) 24h drug treatment at little to no effect on locomotor coordination (climbing), and while 200μg/ml drug treatment produced

measurable climbing defects (**Appendix B: S1**), it did not noticeably affect baseline courtship activity.

For these experiments, virgin male flies were raised on standard food, and switched to food containing RU486 the day before training. Half of each group received a heat shock treatment to induce dCREB2a expression. At low concentrations of drug (5μg/ml), dCREB2-a induction did not produce courtship suppression in any group of flies (**Figure 4.2.4 A**). At a higher concentration of drug (200μg/ml), 3h of training unexpectedly produced robust courtship suppression memory in both genotypes in the absence of heat shock induction (**Figure 4.2.4 B**). When hs-dCREB2a was induced by heat shock, memory was abolished in the control dCREB2-a-GS-ΔNC group (**Figure 4.2.4 B**). However, significant memory was restored in the dCREB2-a -GS-ΔC group (**Figure 4.2.4 B)**. Consistent with our earlier results (**Figure 4.2.3 B**), heat shock induction of dCREB2-a produced only trend towards courtship suppression memory in dCREB2-a-GS-ΔC flies that received 3h training without drug induction.

Taken together, it appeared that at high (but not low) concentrations, drug feeding itself was sufficient to produce memory enhancement, even in the absence of dCREB2-a induction. Furthermore, dCREB2-a induction itself appeared to block this effect. However when excitability was lowered by dORKΔC expression, memory was rescued, either by revealing an enhancement effect of dCREB2-a induction, or by counteracting an anti-drug enhancement effect of dCREB2-a induction.

To directly test whether the drug treatment alone was sufficient to enhance memory formation in these flies, regardless of excitability modulation or dCREB2

induction, we compared 807-GS-ΔNC flies pre-fed with 200ug/ml drug to flies pre-fed with control food. Again, 200ug/ml drug alone prior to 3h training was sufficient to produce LTM (**Figure 4.2.5 A**). Next, we wanted to directly test for an interaction between drug enhancement and CREB. Therefore, we compared memory between uninduced and induced groups of *hs*-dCREB2a flies receiving 3h training. While we did not observe significant courtship memory in the un-induced group ($p=0.06$), the trend towards courtship suppression was abolished following heat shock induction (**Figure 4.2.5 B**). Heat shock alone did not preclude drug enhancement in our control genotype (**Figure 4.2.5 C**). Taken together, these data support the conclusion that drug treatment alone can facilitate memory, gain-of-function in dCREB2 can facilitate memory, and that there is an oppositional interaction between these two factors.

Discussion

In this study, we have identified four factors that appear to regulate sub-threshold memory formation: crowding, excitability, dCREB2 activity, and high dose RU486 treatment. Furthermore, our data suggest that there interactions between several of these factors.

First, we find that moderate crowding alone can facilitate courtship memory formation, but not high levels of crowding (**Figure 4.2.1**). While it is difficult to determine conclusively whether this is a result of social enrichment, or of stress, this result is consistent with inverted-U models of the effect of stress on cognitive performance and memory formation (Salehi et al., 2010; Yerkes and Dodson, 1908). Interestingly, both social enrichment and stress have been suggested to positively regulate CREB activity (Boer et al., 2007; Ganguly-Fitzgerald et al., 2006; Wang et al., 2008). However, whether or not this improved memory performance is a dCREB2-mediated effect, or whether there is a direct interaction between crowding and dCREB2-a mediated enhancement remains to be tested.

Consistent with our previous results, we also find that gain-of-function in dCREB2 activity by heat shock-driven expression of dCREB2-a enhances long-term memory following 5h training (**Figure 4.2.3 C**). We extend these results by characterizing the training threshold for dCREB2-mediated enhancement, and find that dCREB-a induction is not sufficient to produce memory following 2h or 3h training (**Figure 4.2.3 A, B**). This differs somewhat from enhancement for olfactory classical conditioning, where dCREB2 activator induction can produce LTM from a single

training trial. Normally, 1h training is sufficient to produce short term courtship suppression, lasting a little over 1h (Siegel and Hall, 1979). However, we do not observe equivalent dCREB2-a enhancement with limited training conditions for courtship conditioning, suggesting that in courtship conditioning, enhancement is a more graded effect. This may reflect the underlying flexibility regarding which neural substrates support courtship memory (Griffith and Ejima, 2009), and may explain why enhancement for courtship conditioning is less sensitive to other modulating factors.

Our data also suggest, quite surprisingly, that under our experimental conditions 200μg/ml RU486 feeding alone facilitates courtship suppression. This effect is more powerful than dCREB2 activator induction, and seems to be sufficient to produce robust courtship suppression from 3h training. *Drosophila* do not express progesterone receptor, and there are no close evolutionary homologs that are natural candidates for RU486 action (Brody, 1997). However, there are several types of possibilities that could explain the effect we see. The first is that there is a non-specific pharmacological effect of the drug that regulates courtship behavior, or memory formation. Notably, the steroid hormone ecdysone regulates long-term courtship memory, and under certain conditions administering its metabolite 20E can enhance courtship memory (Ishimoto et al., 2009). Another possibility is that there may be a feeding effect of the drug, where drug administration results in a suppression of feeding behavior. Food deprivation itself can produce single trial memory for aversive olfactory classical conditioning (Hirano et al., 2013). The GeneSwitch system is quite commonly used for inducible gene expression in *Drosophila*, including in memory studies, and is widely assumed to be inactive in flies.

Therefore, it is worthwhile to understand the underlying reason for RU486 enhancement, and future studies are warranted.

Interestingly, regardless of the mechanism it appears that dCREB2-a induction precludes the enhancement effects of the drug, and vice versa, suggesting there may oppositional or homeostatic effects (**Figures 4.2.4 B, 4.2.5B**). Given the baseline effect of RU486 feeding, exploring the interactions between dCREB2 and excitability in memory enhancement are best done with another inducible system. It is difficult to make firm conclusions regarding the relationship between excitability in memory enhancement based on these experiments. However, we do see some indications for an underlying interaction. While a drug effect alone could explain the courtship suppression in *elav*-GeneSwitch;UAS-NaChBac flies at 200μg/ml (**Figure 4.2.2**), it does not appear that a 5μg/ml dose of RU486 is sufficient to produce memory enhancement (**Figure 4.2.4 A**). Therefore, we think it is possible that courtship suppression at 5μg/ml in *elav*-GeneSwitch;UAS-NaChBac flies is due to increased excitability (**Figure 4.2.2**), which would explain the bi-model dose effect. Therefore, we believe that mild increases in excitability may indeed produce memory enhancement. Similarly, though in combination *hs*-dCREB2a and RU486 do not produce memory enhancement, enhancement is rescued when overall neuronal excitability is decreased (**Figure 4.2.4 B**). A thorough dose-response characterization for RU486 in wild type flies would help to strengthen these conclusions, as would the use of another inducible system, such as $Gal80^{ts}$.

Regardless of exact mechanisms, it does seem to be the case that different factors that are capable of facilitating memory formation (RU486 feeding, crowding, dCREB2, excitability, time of day) are not additive, and in fact are more likely to be obstructive. Our belief is that this indicates that there are common pathways to memory enhancement, that there are particular set-points that are involved in gating memory formation, and that these pathways and set-points are likely to be homeostatically regulated. By this model, inputs onto CREB imposed by different pathways and physiological processes, (e.g. metabolic state, circadian state, stress, other behavioral experience), can be negotiated in setting the threshold for memory formation. We hope these studies are an initial step towards understanding the complexities of memory enhancement, and this may ultimately help to guide therapeutic strategies.

4.3 ***Immediate Questions and Suggested Future Directions***

1. How do specific crowding conditions map onto dCREB2-mediated enhancement?
2. Does NaChBac overexpression enhance courtship memory using a different induction system?
3. If so, does NaChBac overexpression occlude dCREB2-a enhancement?
4. Does dORK overexpression facilitate dCREB2-a enhancement using a different induction system?
5. What is the underlying cause of RU486 enhancement?

While the experiments described in this chapter, and the above questions, are a reasonable first step in describing the factors that regulate enhancement, in the long

term, better understanding enhancement mechanisms will require more precisely targeted manipulations. The circuitry underlying even simple forms of memory is very complicated (Chapter 3), but one initial approach could be to test whether courtship memory can be enhanced by dCREB2-a induction in particular brain areas immediately prior to training. While courtship memory can be supported by different mechanisms, targeted dCREB2 activation may drive memory formation towards those particular areas. Independent of genetic manipulation, courtship memory can also be directed towards particular brain areas by excluding particular sensory inputs. For example, training under dim red light conditions will make courtship memory depend more heavily on the mushroom body (Joiner and Griffith, 1997). If particular neural substrates can be identified that are sufficient to support memory enhancement, these experiments can be refined to include targeted manipulation in different subsets of neurons. Another strategy is to identify particular dCREB2 target genes that are sufficient to support enhancement, which may allow for more targeted strategies. Once the parameters regulating enhancement are better defined, ultimately these principles can be applied to olfactory classical conditioning, where memory mechanisms are better understood.

Figures and Legends

Figure 4.2.1

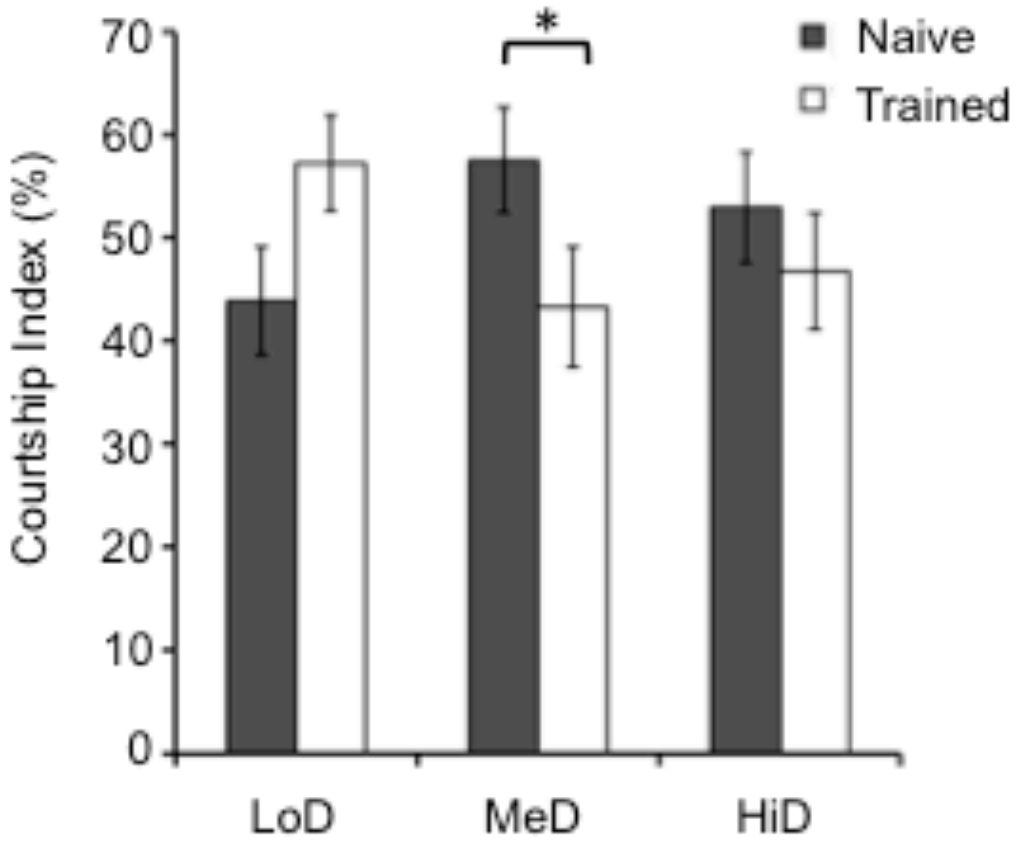

Figure 4.2.1: Moderate crowding enhances 3-day courtship suppression memory. 3-day memory is indicated for groups receiving 3h of crowding treatment immediately prior to 5h conditioning. Mean courtship index is indicated for naïve (grey bars) and trained (white bars) groups. Experimental groups are indicated below the graph, representing low density (LoD), medium density (MeD), or high density (HiD) conditions prior to training. Error bars indicate ± SEM. Within each experimental group, (*) indicates significant courtship suppression ($p<0.05$), no star indicates no significant courtship suppression ($p\geq0.05$).

Figure 4.2.2

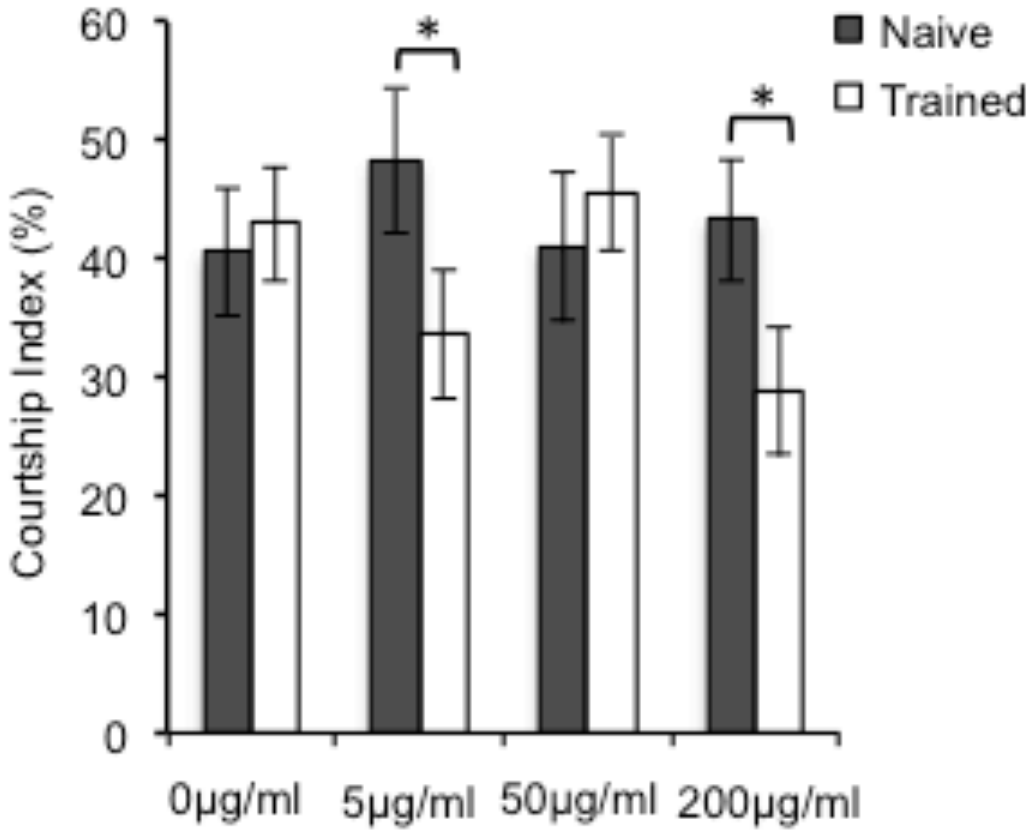
Naive
Trained
Courtship Index (%)
60
50
40
30
20
10
0
*
*
0µg/ml
5µg/ml
50µg/ml
200µg/ml

Figure 4.2.2: Enhancement of LTM with RU486-induced NaChBac overexpression. Mean 3-day courtship index ± SEM for naïve (grey bars) and trained (white bars) groups. *elav*-GeneSwitch/UAS-NaChBac flies were placed on food containing varying concentrations of RU486 (0, 5, 50, or 200ug/ml) to activate NaChBac expression for 24h before 4h training. Within each experimental group, (*) indicates significant courtship suppression ($p<0.05$), no star indicates no significant courtship suppression ($p\geq0.05$).

Figure 4.2.3

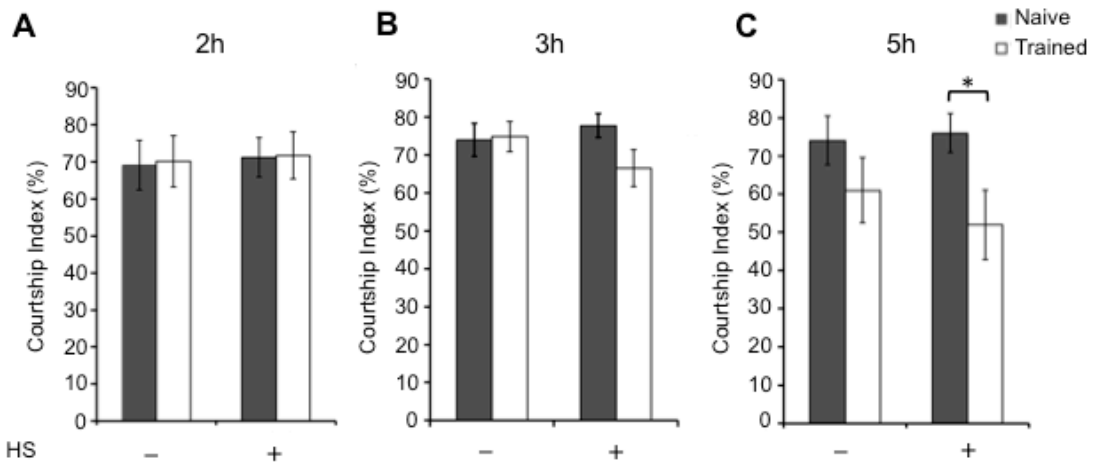
A
2h
B
3h
C
5h
Naive
Trained
Courtship Index (%)
90
80
70
60
50
40
30
20
10
0
HS
–
+
*

Figure 4.2.3: dCREB2 activator enhancement of courtship memory requires 5h training, 3-day memory is indicated for *hs*-dCREB2a flies receiving a heat shock treatment (+) to activate dCREB2-a expression, or handling alone (-) prior to 2h (A), 3h (B), or 5h (C) conditioning. Mean courtship index ± SEM is plotted for naïve (black bars) and trained (white bars) groups. (A) dCREB2 activator induction prior to training does not enhance courtship suppression memory following 2h training. (B) dCREB2-activator induction prior to 3h training does not produce significant courtship suppression memory. (C) dCREB2-activator induction prior to 5h training enhances courtship suppression memory (*) indicates significant courtship suppression ($p<0.05$), no star indicates no significant courtship suppression ($p\geq0.05$).

Figure 4.2.4

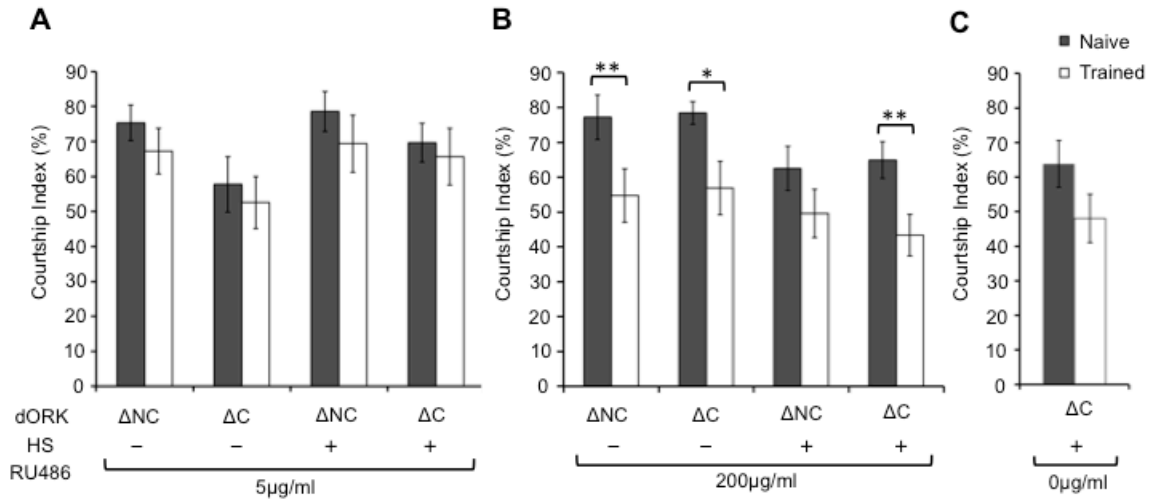
A
B
C
Naive
Trained
Courtship Index (%)
dORK
HS
RU486
ΔNC
ΔC
5μg/ml
200μg/ml
0μg/ml
**
*

Figure 4.2.4: Complex modulation of memory by neuronal excitability, dCREB2, and RU486 feeding. 3-day courtship indices are indicated for *hs*-dCREB2a;elav-GeneSwitch/UAS-dORK flies. For each experimental group, trained flies (white bars) received 3h training, while naïve flies (black bars) received 3h sham training. Experimental groups carrying a conducting (ΔC) or non-conducting (ΔNC) version of the dORK transgene are indicated immediately below the graph. Experimental group receiving a heat shock treatment (+) to induce dCREB2-a expression or a sham treatment (-) are also indicated below the graph, as is RU486 dose used for each experiment: (A) flies were pre-fed for 24h with 5μg/ml RU486, (B) flies were pre-fed for 24h with 200μg/ml RU486, (C) flies were pre-fed for 24h with vehicle alone. Stars indicate significant courtship suppression in naïve compared to trained groups (*$p<0.05$, **$p<0.01$), no star indicates no significant courtship suppression in trained compared to naïve groups ($p\geq0.05$).

Figure 4.2.5

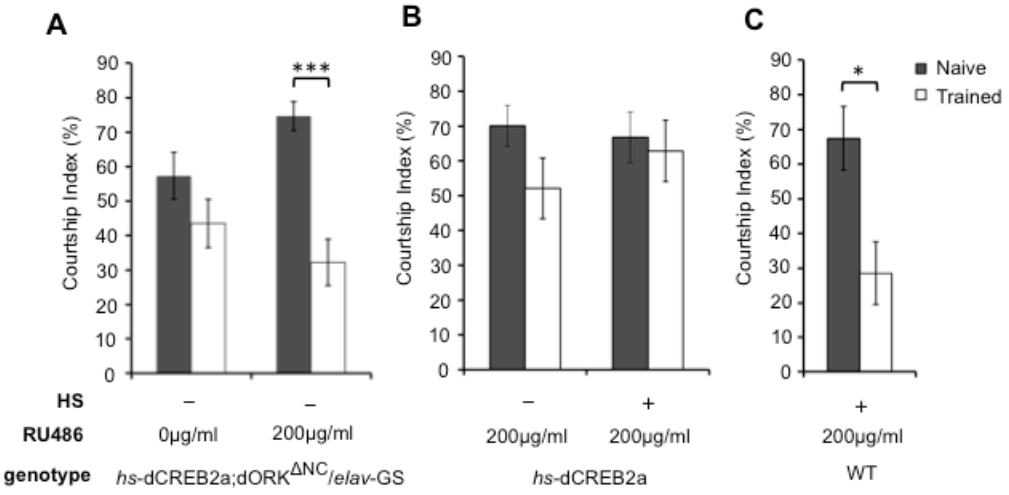
A
B
C
Courtship Index (%)
***
*
Naive
Trained
HS
RU486
0µg/ml
200µg/ml
genotype
hs-dCREB2a;dORK^ΔNC/elav-GS
hs-dCREB2a
WT

Figure 4.2.5: RU486 feeding enhances courtship suppression. 3-day courtship indices are indicated for *hs*-dCREB2a;elav-GeneSwitch/UAS-dORK flies. For each experimental group, trained flies (white bars) received 3h training, while naïve flies (black bars) received 3h sham training. Experimental groups are indicated as follows: heat shock treatment (+), or a sham treatment (-), and pre-feeding RU486 dose are indicated below the graph (A) *hs*-dCREB2a;elav-GeneSwitch/UAS-dORKΔNC flies were pre-fed for 24h with 200μg/ml RU486 or vehicle alone (B) *hs*-dCREB2a flies were pre-fed for 24h with 200μg/ml RU486, and received heat shock treatment (+) or not (-) for 30m prior to training to induce activator expression (C) Heat shock alone without dCREB2-a induction does not block drug enhancement. CRE-dEGFP (WT) flies were pre-fed for 24h with 200μg/ml RU486, and received a heat shock treatment. Stars indicate significant courtship suppression in naïve compared to trained groups (*$p<0.05$, ***$p<0.001$), no star indicates no significant courtship suppression in trained compared to naïve groups ($p\geq0.05$).

Acknowledgements: Chapter 4

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Chapter 5: Future Directions

This dissertation was driven by several key questions: 1) How is CREB activity organized over time across the brain? 2) How is CREB activity modulated by memory formation over time, and across the brain? 3) What mechanisms underlie CREB-mediated memory enhancement, and why does it only work sometimes? While we have taken meaningful steps towards answering these questions, all of them are still only partially resolved. Building on the work presented here, there is room to move toward better answers and broader questions.

How is CREB activity organized over time across the brain? (Chapter 2)

In the work presented here, we characterize synchronous circadian activity patterns of dCREB2 *in vivo* in central and peripheral oscillating tissues. What drives these oscillations? What is their functional significance? Neither of these are easy questions to answer. However, a reasonable first question is whether components of the core oscillators are required intracellularly for transcription factor oscillations. Our data show, unsurprisingly, that dCREB2 and NF-κB circadian oscillations require an intact circadian system in general. However, we could also manipulate core clock genes only within a particular set of 'peripheral' cells such as MB neurons, and ask whether MB-specific oscillations are blocked. If this is the case, a way to approach the functional significance might be to impair rhythmicity in particular neurons, and ask what the functional consequences are. What happens when we interfere with these oscillations in

dCREB2 activity in the MB? Is memory maintenance impaired? Is cellular health impaired?

How is CREB activity modulated by memory formation over time, and across the brain? (Chapter 3)

The GAL4-UAS system, in combination with a conditional reporter strategy has greatly expanded the power of *in vivo* reporters by allowing us to delineate the subsets of neurons that we measure. This has allowed us to make a number of observations about the pattern of dCREB2 activity, and how it might fit in to mechanisms of systems processing. However, some of the biggest limitations that exist in our reporter system are driven primarily by the layering of the GAL4 system. On a technical level, the sensitivity of our reporter system is constrained by the low-level leaky expression from the UAS-FLP transgene. This sets a manageable, but real limit on the signal to noise ratio of this system that has prevented us from measuring reporter activity in very small cell populations (for example, the single pair of DAL neurons). Because the CRE-F-luc design has no leaky expression in and of itself, the growing availability of direct enhancer trap FLP lines, as well as the ability to engineer more targeted FLP lines are likely to overcome some of these issues.

On a scientific level, by use of the GAL4 system, we have selected our measurement populations based on anatomical, rather than functional criteria. However, in flies and in other systems, specific memories are sparsely encoded. By comparing forward to backward control groups, we have tried to experimentally isolate

trace-specific changes in activity. However, this approach has inherent limitations. In the memory field, there is a growing trend toward memory trace selection for transgenic manipulation. This trace selection can be based either on activity-dependent gene expression, or on artificial bias imposed by sparse CREB overexpression, or (Han et al., 2009; Kim et al., 2014; Reijmers and Mayford, 2009). By layering this strategy in with the CRE-F-luc reporter, there is potential to build a system that approaches the holy grail for these studies of memory formation: real-time spatio-temporal monitoring of memory-dependent gene expression both pre- and post-memory formation, combined with spatio-temporal control of genetic manipulation. For example, we could combine a *hs*-F-dCREB2-activator that we have developed with our CRE-F-luci reporter, drive the memory trace towards a particular subset of neurons by activating dCREB2, then ask how dCREB2 is modulated over time within that subset of neurons.

By layering trace selection, and activity-dependent tagging onto our reporter approach, there are several major questions across the memory field that are currently technically out of reach in mammals, but are now potentially within reach in *Drosophila*.

> *"…is it possible that memory training actually entrains memory-specific neuronal ensembles in the hippocampus? This question could be addressed by tracking individual neurons after training using a bioluminescent reporter that is specific for training-activated neurons. Using such a reporter, it might be possible to determine whether circadian oscillations in training-activated neurons are amplified or phase shifted. Such detailed analysis would be challenging, but would shed light on the temporal organization of memory encoding and temporary maintenance in the presence of already oscillating pathways."*
>
> *Eckel-Mahan and Storm, 2009*

> *"…the studies described above artificially manipulated the level of CREB function in a subset of neurons to forcibly bias CREB levels in amygdala circuit. However, it remains unclear whether different cells in amygdala network actually have different levels of endogenous CREB function at any particular moment; if so, how are they generated and coordinated? A real-time reporter system capable of reflecting the functional level of endogenous CREB in a defined cell population would help address this question."*
>
> *Kim et al., 2013*

What mechanisms underlie CREB-mediated memory enhancement, and why does it only work sometimes? (Chapter 4)

There are several mechanisms through which CREB gain-of-function might enhance memory formation. Well-supported canonical (synaptic plasticity) models of the role of CREB in memory formation suggest that CREB functions in memory formation by providing the required machinery for consolidation of synaptic plasticity. Therefore, one possibility is that CREB reduces the threshold for behavioral memory by bypassing the requirement for *de novo* gene expression that supports synaptic plasticity. Elegant studies demonstrating synaptic tagging suggest that this is a viable explanation (Frey and Morris, 1997). A second possibility is that gain-of-function in CREB activity also leads to a state in which long-term memory is able to form by increasing excitability, thereby lowering the input threshold for memory formation. Indeed, in mammals, artificially increasing neuronal excitability in distributed neuronal populations enhances memory formation, closely mimicking the effect of CREB overexpression (Yiu et al., 2014). A synaptic-only enhancement model is difficult to reconcile with the conditional and obstructive memory enhancement effects that we see. By this model, CREB activity facilitates memory formation by supplying synaptic

proteins that are then captured during sub-threshold training. If this is the case, activating CREB activity before training should always allow for sub-threshold memory formation, which does not occur. However, if enhancement is excitability-mediated, it is easier to see how other upstream or downstream factors that regulate excitability might occlude this effect.

If CREB plays dual roles in synaptic plasticity and excitability, and is both upstream and downstream of excitability, how can we test an excitability-related role experimentally without conflating these processes? By thoroughly characterizing enhancement effects on a molecular level, we may be able to tease apart these two roles. Ultimately, the best approach might be to identify dCREB2 target genes that regulate intrinsic excitability or synaptic plasticity, and ask which combinations are necessary or sufficient for different components memory formation.

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Frey, U., and Morris, R.G. (1997). Synaptic tagging and long-term potentiation. Nature *385*, 533–536.

Han, J.-H., Kushner, S.A., Yiu, A.P., Hsiang, H.-L.L., Buch, T., Waisman, A., Bontempi, B., Neve, R.L., Frankland, P.W., and Josselyn, S.A. (2009). Selective erasure of a fear memory. Science *323*, 1492–1496.

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Appendix A

This appendix includes supplemental information for Chapter 2

Supplemental Information (Chapter 2.2)

Tanenhaus AK, Zhang J, Yin JCP (2012) *In vivo* circadian oscillation of dCREB2 and NF-κB activity in the *Drosophila* nervous system. 7(10):e45130

Supplemental Figures

Figure S1

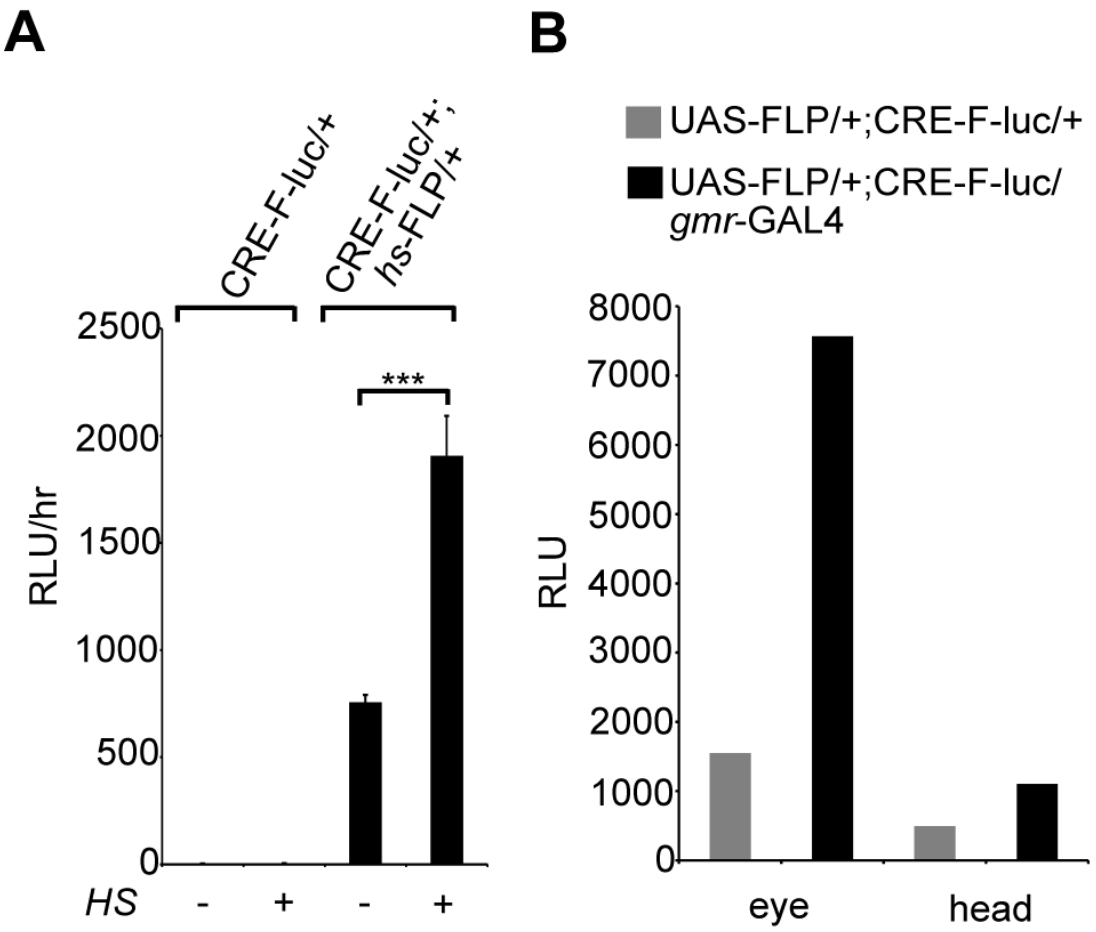

Figure S1. Supplemental CRE-F-luc reporter validation. (A) CRE-F-luc reporter activity is FLP-dependent. Reporter activity (Y-axis, relative light units) is plotted as a function of genotype (singly [CRE-F-luc] or doubly transgenic flies [*hs*-FLP/+;CRE-F-luc/+] and treatment (heat shock HS+ or not, HS-). The histogram bars indicate mean hourly counts over a 3-day window (n=24). (B) Anatomical specificity of *gmr*-GAL4 driven reporter. *In vitro* luciferase activity measured in extracts made from dissected eye or remaining head tissue (n=5). The relative light units (Y-axis) are plotted as a function of the genotype (shown in gray [UAS-FLP/+; CRE-F-luc/+ or black [UAS-FLP/gmr^{long}-GAL4; CRE-F-luc/+]) or tissue source. (Error bars = S.E.M, **** = $p<0.0001$).

Figure S2

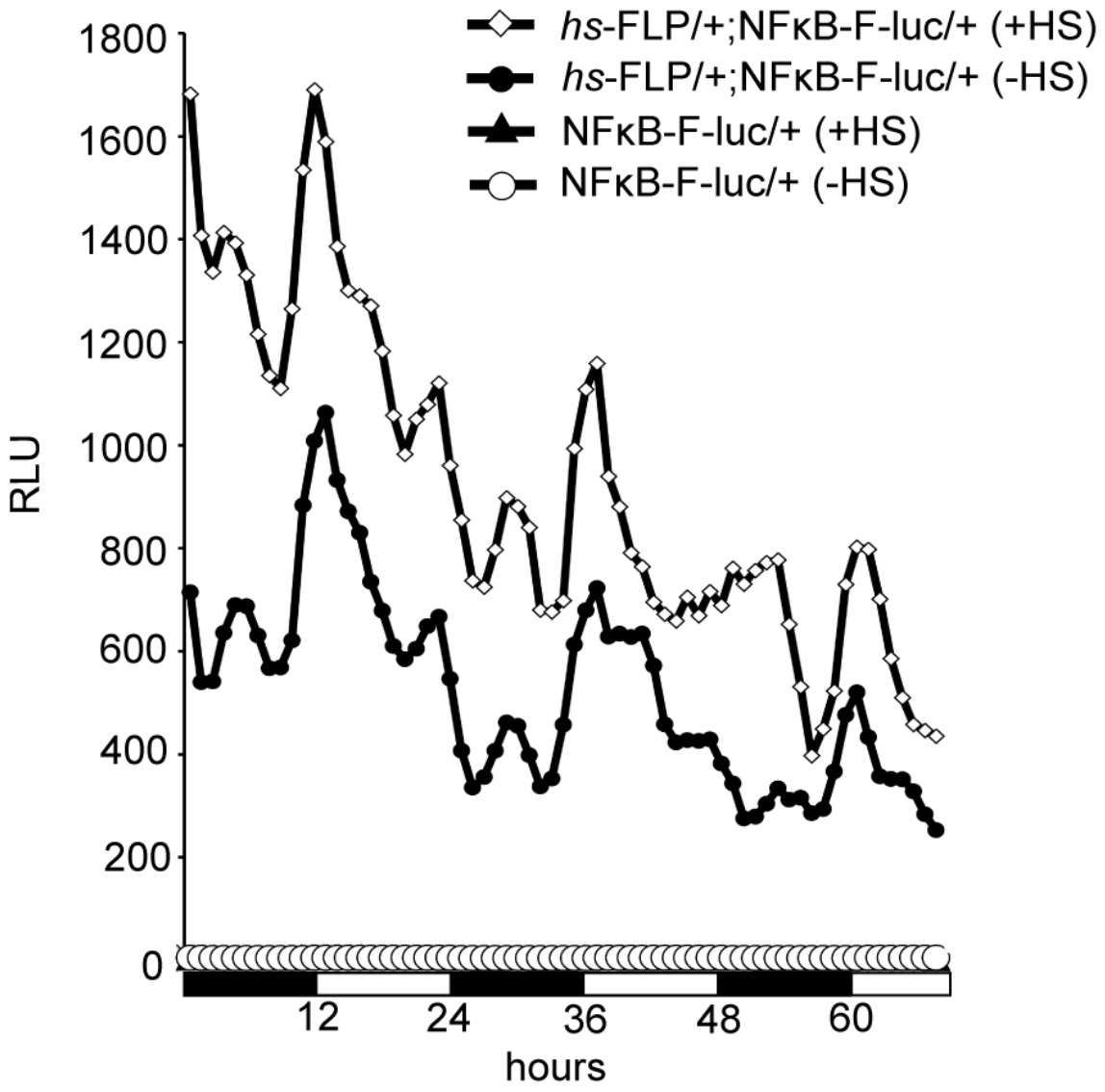
hs-FLP/+;NFκB-F-luc/+ (+HS)
hs-FLP/+;NFκB-F-luc/+ (-HS)
NFκB-F-luc/+ (+HS)
NFκB-F-luc/+ (-HS)
RLU
1800
1600
1400
1200
1000
800
600
400
200
0
12
24
36
48
60
hours

Figure S2. NF-κB reporter activity oscillates over the day-night cycle. FLP-activated NF-κB-F-luc activity. Singly (NFκB -F-luc) or doubly transgeneic flies (*hs*-FLP/+; NFκB -F-luc /+) are maintained under 12:12 LD conditions. Flies are exposed to heat-shock (+HS) or not (-HS), and measured for *in vivo* luminescence. The relative luminescence is plotted as a function of time, with daytime (white bars) and nighttime (black bars) conditions indicated below the graph. Each data point represents the average of 24 flies.

Figure S3

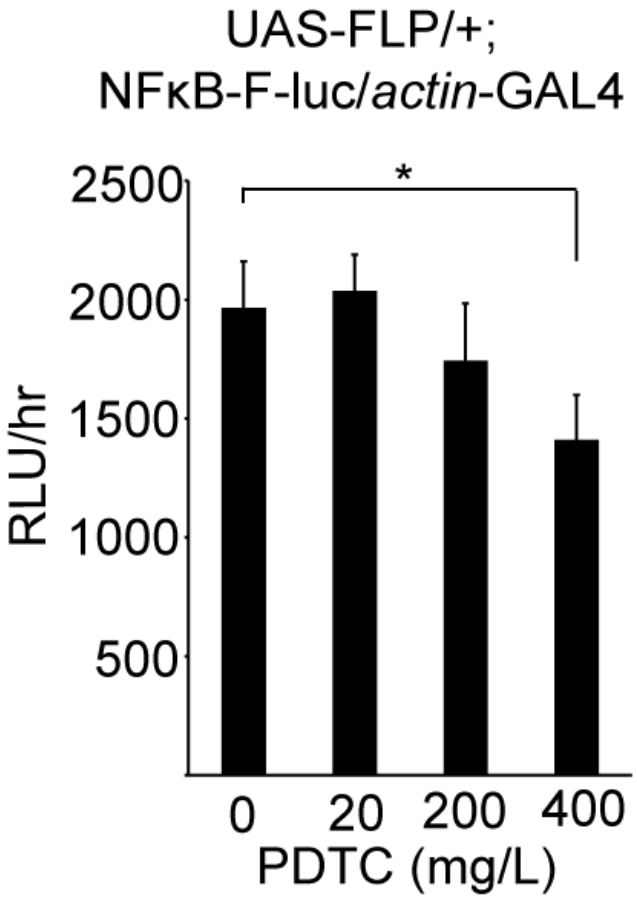
UAS-FLP/+;
NFκB-F-luc/actin-GAL4
2500
2000
1500
1000
500
RLU/hr
*
0
20
200
400
PDTC (mg/L)

Figure S3. PDTC inhibition of NFκB-F-luc reporter activity persists for 24 hours at high doses. The relative luminescence is plotted with respect to the PDTC dose fed to flies. Triply transgenic flies (UAS-FLP/+;NF-kB-F-luc/*actin*-GAL4) were fed different dosages of PDTC for 24 hours and then measured for luminescence 1h after the end of feeding. Reporter activity is pooled over 1 day following 24h PDTC feeding (n=24 for each group) (Error bars = S.E.M, *p<.05).

Figure S4

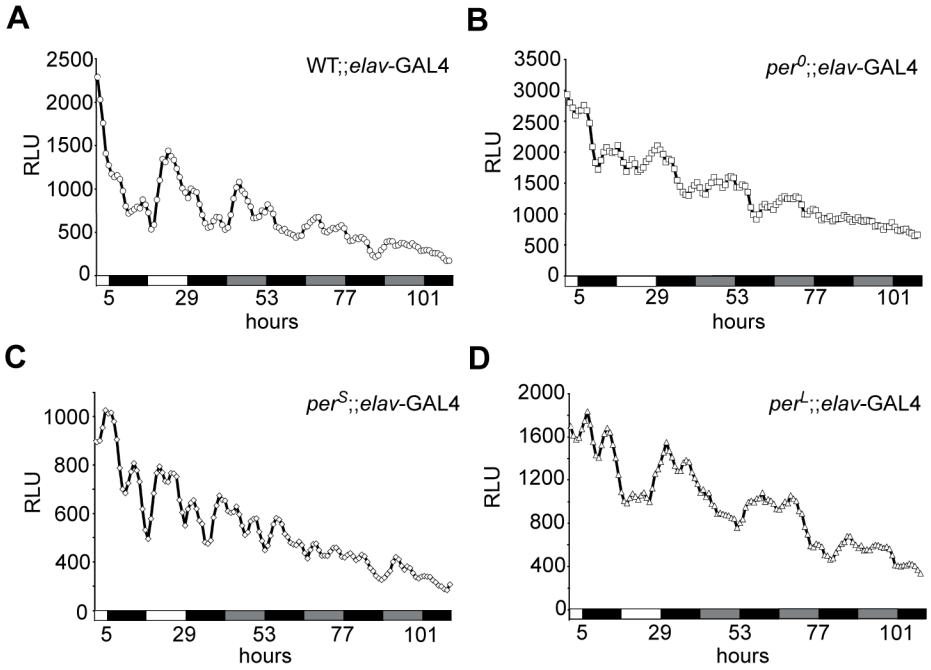
A
WT;;elav-GAL4
RLU
2500
2000
1500
1000
500
0
5
29
53
77
101
hours
B
per0;;elav-GAL4
RLU
3500
3000
2500
2000
1500
1000
500
0
5
29
53
77
101
hours
C
perS;;elav-GAL4
RLU
1000
800
600
400
0
5
29
53
77
101
hours
D
perL;;elav-GAL4
RLU
2000
1600
1200
800
400
0
5
29
53
77
101
hours

Figure S4. Neuronal CRE-F-luc reporter cycling is modulated in *per* mutants. Neuronal reporter activity is plotted over time as flies are shifted from light:dark to constant darkness. For all of these panels, the same transgenes (UAS-FLP/+; NF-kB-F-luc/ *elav*-GAL4) exist in all flies, but the flies are examined in a wild type (A), per^0 (B), per^S (C) or per^L (D) genetic background.

Figure S5

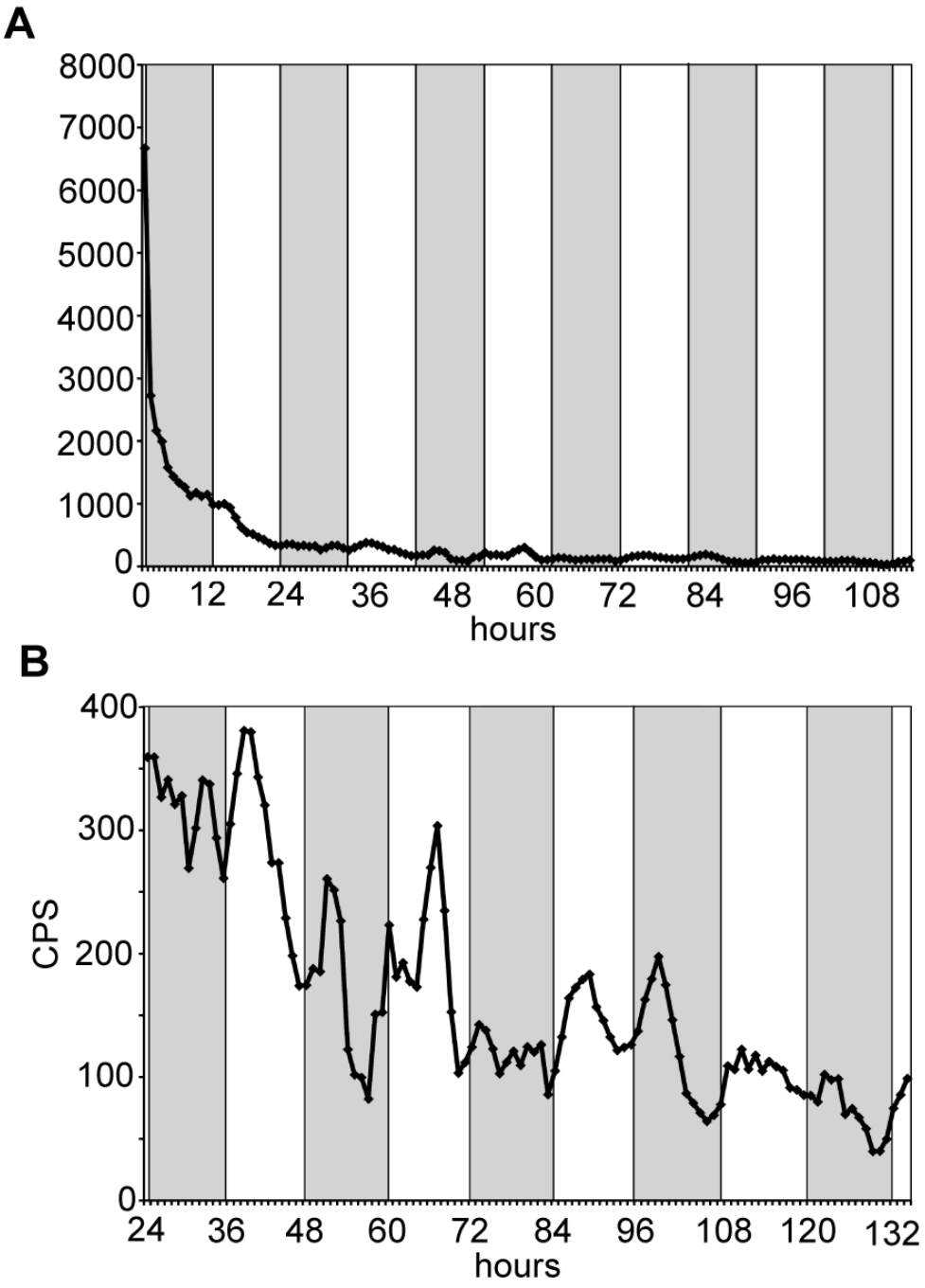
A
8000
7000
6000
5000
4000
3000
2000
1000
0
0 12 24 36 48 60 72 84 96 108
hours
B
400
300
200
100
0
CPS
24 36 48 60 72 84 96 108 120 132
hours

Figure S5. Oscillations in CRE-luc reporter activity persist after substrate removal. (A) Reporter activity (in relative light units, RLU) in CRE-luc reporter flies is plotted as a function of time, starting 1h after the transfer from luciferin to non-luciferin food. Light conditions are indicated by white boxes (daytime) and grey boxes (nighttime). (B) The same data as in (A), with the first day excluded.

Supplemental Table 1: dCREB2 Reporter Driver Screen

Driver	Expression Pattern	Significant Signal	Reporter activity (%background)	Daytime peak	Nighttime peak
hs-GAL4	Global	Yes	775	-	-
actin-GAL4	Global	Yes	1266	✓	✓
ok107-GAL4	Mushroom body: all lobes	Yes	155	✓	✓
c739	Mushroom body: αβ lobes	Yes	873	✓	✓
c42	Ellipsoid body: R2/R4m neurons	Yes	817	✓	✓
c232	Ellipsoid body: R3/R4d neurons	Yes	289	✓	✓
elav^{c155}-GAL4	Pan-neuronal	Yes	680	✓	✓
repo-GAL4	Pan-glial	Yes	1389	✓	✓
mz0709-GAL4	Ensheathing Glia	Yes	1249	✓	✓
alrm-GAL (2)	Astrocytic glia	Yes	176	✓	✓
alrm-GAL4 (3)	Astrocytic glia	Yes	372	✓	✓
npf-GAL4	Neuropeptidergic	No	-	-	-
gmr-GAL4	Pan-eye	Yes	948	✓	✓
gmr^{long}-GAL4	Pan-eye	Yes	574	✓	✓
ninaE-GAL4	Pigment cells (photoreceptors)	Yes	330	✓	✓

Significant signal (RLU/hr, $p<0.05$) for driver/UAS-FLP/CRE-F-luc flies (reporter)

compared to UAS-FLP/CRE-F-luc flies (background) *in vivo* under LD conditions.

Average reporter activity (RLU/hr) is expressed as a percentage of background activity.

Check marks indicate the presence of an activity peak during the daytime (ZT=0-12) or nighttime (ZT=12-24).

Supplemental Methods

Eye Dissection

Heads were removed from female flies and eyes were dissected in cold PBS. The remaining head tissue was collected and assayed separately. Luciferase activity in dissected tissue was measured using the SteadyGlo Luciferase Assay System (Promega).

Supplemental Luciferin Pre-Feeding

CRE-luc flies were entrained to a 12:12 LD cycle for 5 days, and transferred to luciferin food (5mM luciferin, 1% agar, 5% sucrose) for 3h. After pre-feeding, flies were loaded into 96-well plates containing luciferin-free food (1% agar, 5% sucrose), and subsequent reporter activity was recorded.

Supplemental fly lines for dCREB2 reporter activity screen

The *c739*, *npf*-GAL4, *NinaE*-GAL4, *pdf*-GAL4, *Tdc1*-GAL4, *Tdc2*-GAL4, *gmr*-GAl4, and *repo*-GAL4 lines were obtained from the Bloomington Stock Center. The c42, c232 lines were kindly provided by V. Jayaraman. The *mz0709*-GAL4, and *alrm*-GAL4 lines were kindly provided by Marc Freeman. GAL4 driver lines or wildtype lines were crossed to UAS-FLP;CRE-F-luc flies, and tested for 3-4 days under 12:12 LD conditions for *in vivo* luciferase activity. Significant signal vs. baseline was determined by comparing hourly light count between triply transgenic GAL4 driver/UAS-FLP/CRE-F-luc flies and concurrently run doubly transgenic UAS-FLP/CRE-F-luc flies. The presence of daytime (ZT=0-12) or nighttime (ZT=12-24) peaks was scored visually.

Supplemental Information (Chapter 2.3)

Figure S7

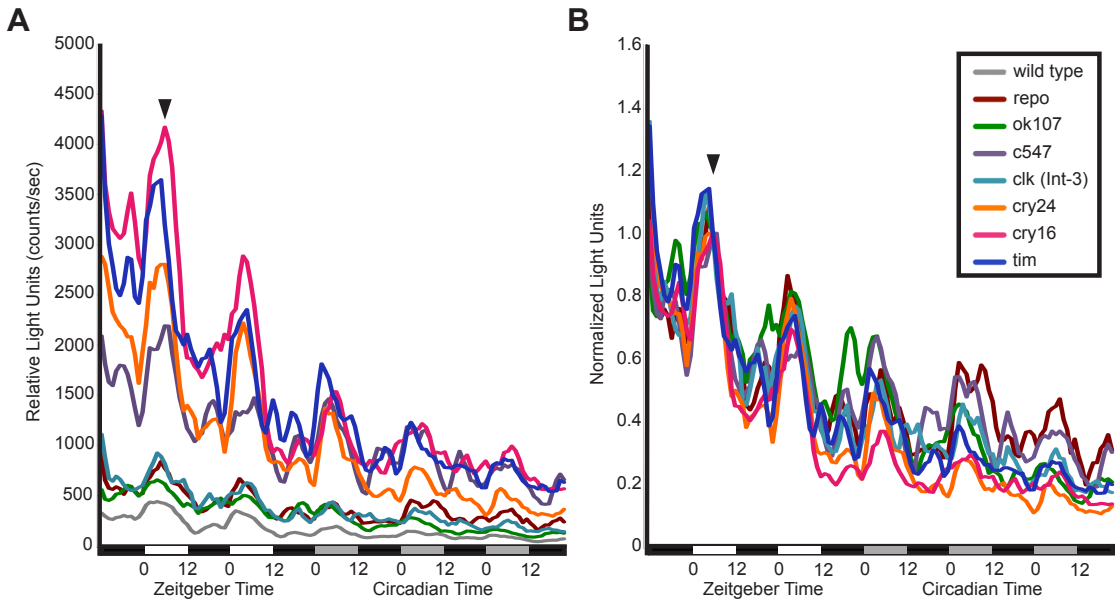

Figure S7: dCREB2 transcriptional activity has a similar pattern of oscillation across cell populations. (A) Same data presented in section 2.3 Figure 2.3.1, except normalized oscillations are also presented in (B) for better temporal comparison. For each group, each data point presented as relative values with respect to the first daytime peak, indicated by the arrow. Bioluminescence is plotted in multiple fly lines expressing a dCREB2 luciferase reporter under the control of tissue-specific GAL4 drivers. Each line represents the mean reporter activity over time (hours) across flies (n=48) representing the progeny of UAS-FLP;CRE-F-luc transgenic flies crossed with wild type (grey), or GAL4 driver lines: *c547* (red), *ok107* (green), *repo*-GAL4 (purple), *clk(int-3)-GAL4* (teal), *crypGAL4-24* (cry24, orange) *crypGAL4-16* (cry16, pink), *tim*-GAL4 (blue). Each data point represents the average over three hourly time points. Light conditions are indicated along the x-axis first in LD (black: night, white: day), then in DD (black: subjective night, grey: subjective day).

Supplemental Information (Chapter 2.5)

Figure S6

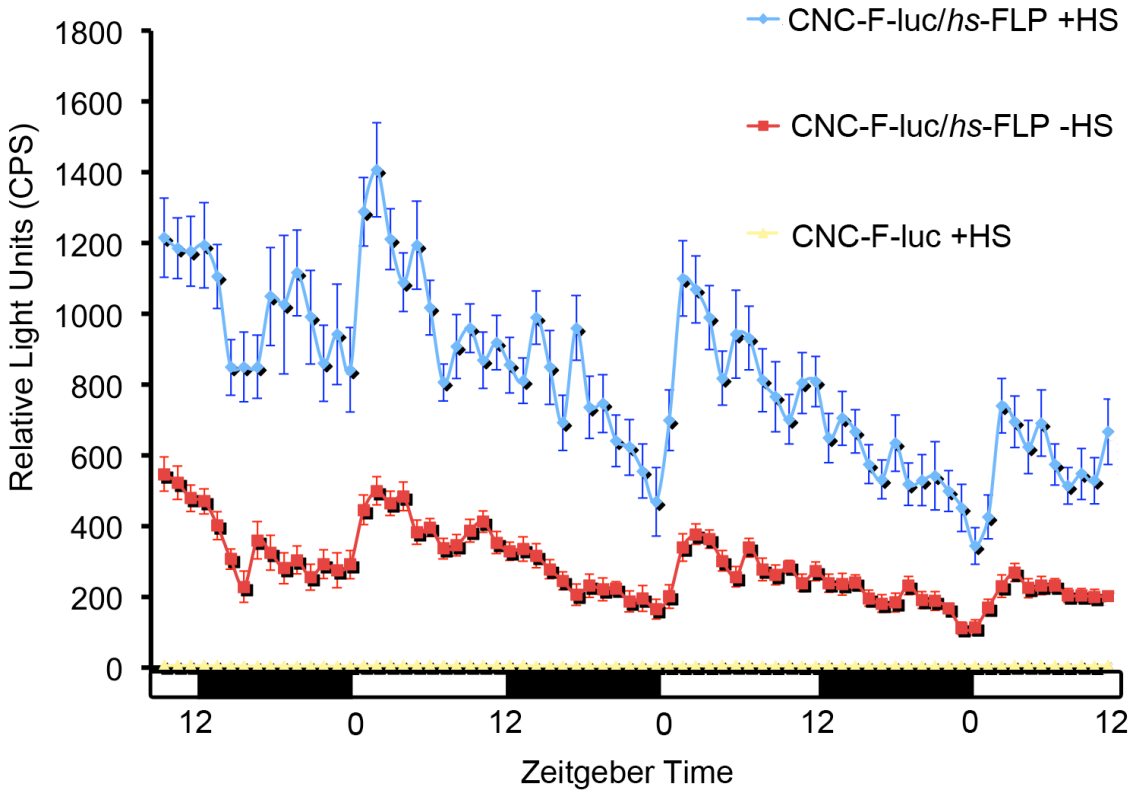

Figure S6. Diurnal Oscillations in a Cap'n'Collar reporter. Mean bioluminescence is plotted over time. Flies carrying CNC-F-luc and hs-FLP transgenes received heat shock treatment (+HS) or not (-HS) 24h prior to loading, or flies carrying CNC-F-luc transgene only received heat shock treatment. The first 24h are excluded from analysis to allow for recovery from anesthesia. Each hourly data point indicates mean ± SEM, n=24 flies. Light (white bars) and dark (black bars) conditions are indicated below the graph.

In vivo luminometer assays were performed as previously described (Tanenhaus et al., 2012), except that 5mM luciferin was used for feeding. The Cap'n'Collar reporter (CNC-F-luc) is identical to previously reported FRT-luciferase reporters (Tanenhaus et al., 2012), except that 3x CNC response elements (3' TCAGCATGACCGGGCAAAAA5') (Sykiotis and Bohmann, 2008) are substituted for the CRE sites.

References

Sykiotis, G.P., and Bohmann, D. (2008). Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. Dev. Cell *14*, 76–85.

Appendix B: Supplemental Figure for Chapter 4

Figure S1

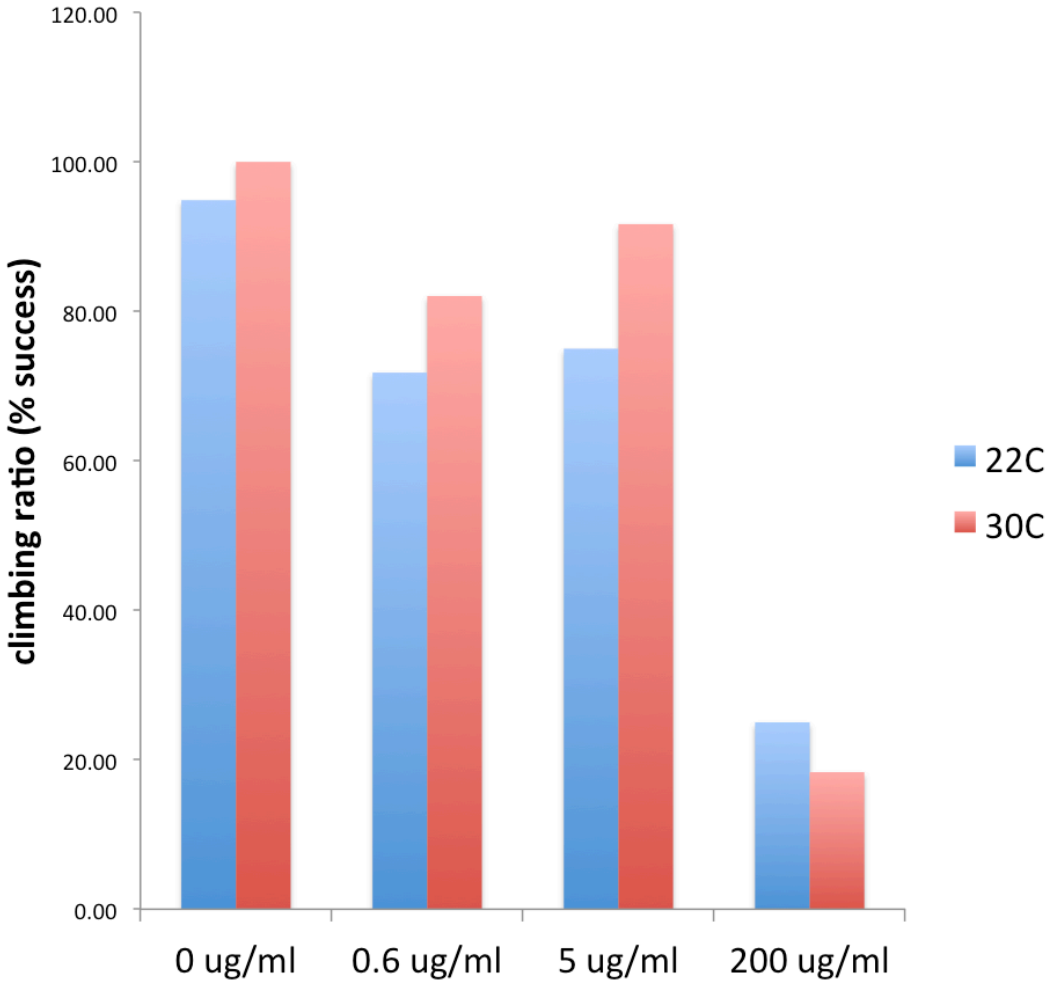

Figure S1. Dose effect of dORKΔC induction on locomotor coordination. Percent climbing success for dCREB2-a;UAS-dORKΔC/*elav*-GeneSwitch flies [(successful flies/unsuccessful flies)x100] is plotted with respect to RU486 concentration following 24h drug feeding. Flies were maintained at 22°C or 30.°C from the beginning of drug feeding to the onset of the climbing assay.