Drosophila CPEB, Orb2, a Putative Biochemical Engram of Long-term Memory

By

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Abstract

How a transient experience creates an enduring yet dynamic memory remains a fundamental unresolved issue in studies of memory. Experience-dependent aggregation of the RNA-binding protein CPEB/Orb2 is one of the candidate mechanisms of memory maintenance. Here, using tools that allow rapid and reversible inactivation of Orb2 protein I find that Orb2 activity is required for encoding and recall of memory. Blocking the Orb2 oligomerization process by interfering with the protein phosphorylation pathway or expressing an anti-amyloidogenic peptide impairs long-term memory. Facilitating Orb2 aggregation by a DNA-J family chaperone, JJJ2, enhances the animal's capacity to form long-term memory. Finally, I have developed tools to visualize training-dependent aggregation of Orb2. I find that aggregated Orb2 in subset of mushroom body neurons can serve as a "molecular signature" of memory and predict memory strength. My data indicate that self-sustaining aggregates of Orb2 may serve as a physical substrate of memory and provide a molecular basis for the perduring yet malleable nature of memory.

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Chapter 1. Introduction

1.1 Memory engram – a historical perspective on the emergence of the concept

A long-pursuing goal in neuroscience is to understand how a transient experience can create a stable yet malleable memory. Dated back to the early 20th century, the German zoologist and evolutionary biologist Richard Semon (1859-1918) formulated the idea of mneme (based on the Greek goddess Mneme, the muse of memory), which describes an internal representation of the external experience. The theory laid a conceptual framework of memory that is still being explored: first, the information is encoded as a physical substrate in the nervous system (input); second, the physical substrate should be conserved after effects of stimulation (storage); and third, the information could be revived when an element resembling a component of the original stimuli was encountered (output) (Semon, 1921). The mnemic trace or "engram" becomes a more widely used term to describe the process of memory encoding, storage and expression. Later the Canadian neurosurgeon Wilder Penfield (1891-1976) further divided the memory engram into two basic components (Penfield, 1968): molecular *"(1) What are the basic protoplasmic alterations that make permanent recording of experience and memory recall possible?"* and cellular/network-based *"(2) How and where does the neurone transaction take place that constitutes the record of experience and makes possible its reproduction or recall?"* These two components represent the physical substrates of engram on different levels and from studies of each of these components individually or their interplay, a more comprehensive picture of memory "engram" started to emerge.

1.2 Memory engram on the network and cellular level

1.2.1 A systemic view of memory engram

The search for memory engram at system and circuit level started long before the advancement of molecular biology. Among the eminent psychologists of the 20th century, Karl Lashley (1890-1958) was originally set out to search for a single physical locus of memory engram in the brain. However a decade later he ended up concluding that memories were not localized in any particular part of brain, rather they were diffused throughout the cortex and functionally redundant (Lashley, 1930, 1931). His conclusion was based on creating lesions on different regions of cerebral cortex and realizing no specific regions of the cortex affect memory retention of the rats performing maze learning behavior (Lashley, 1930). What he initially proposed that learning is an associative connection between neurons or brain regions into a functioning path, although a well-accepted concept nowadays, frustrated him during the rest of his career, due to limited methodology and inadequate knowledge of the nervous system at his time (Bruce, 2001).

Looking back, Lashley's attempt wasn't in vain. His failures suggested that memory engram might be located in other brain regions outside the cortical region, or the engram is sparse or maintained dynamically. Indeed, the seminal studies of the historically famous patient Henry Molaison (Patient H.M.) by Brenda Milner and others, shifted the search for memory engram from cortex to the hippocampus in the medial temporal lobe. H.M. developed severe anterograde amnesia after surgical removal of two thirds of his hippocampi and surrounding tissues, in an attempt to treat epilepsy. His surgeon William Scoville (1906-1984) together with Brenda Milner summarized nine other similar cases and systemically reported the correlation between hippocampi lobectomy and memory disorder (Scoville and Milner, 1957). Subsequent studies in the rats with bilateral hippocampal lesions showed slow learning in maze discrimination task (Douglas, 1967), suggesting an evolutionary conserved role of hippocampus in memory. In the 1960s, implanting microelectrodes to the freely-moving rodents and monitoring the neural activity became possible

(Ainsworth et al., 1969). John O'Keefe (the 2014 Nobel laureate) first applied the technique to the central nervous system and identified hippocampal cells that respond to specific spatial cues, indicating hippocampal cells may encode spatial memory (O'Keefe and Dostrovsky, 1971). It was a revolutionary advance in the study of engram, as visualizing neuronal activity in response to external stimuli became possible and the exploration of engram was no longer only based on lesion study. That there is indeed a physical representation of experience in a specific location in the brain came from Wilder Penfield when he was treating patients with epilepsy. He first reported that stimulation of the temporal lobes could lead to recall of specific memories (Penfield, 1952). This is the first evidence that stimulation of the cells that store the memory can evoke the recall of that experience. Subsequent theoretical (Marr, 1971) and experimental evidence (Squire and Alvarez, 1995; Zola-Morgan and Squire, 1990) led to the current model that episodic memories are rapidly formed in hippocampus by strengthening connections between neurons and then transferred to neocortex for long-term/off-line storage. However, a recent study suggests the memory traces are formed in the hippocampus and cortex simultaneously but the cortical trace remain dispensable for retrieval until months later (Kitamura et al., 2017).

Besides the hippocampus dependent engram of spatial and episodic memories, it also became evident that there are different forms of memory localized in distinct regions of the brain, mediated by a different neuronal ensemble, and overtime can be transferred from one brain region to the other (Eichenbaum, 2016; Squire, 2004). For example, the study of the Pavlovian conditioning with the eyelid response revealed a comprehensive memory trace in the cerebellum (Poulos and Thompson, 2015), and the fear conditioning revealed an engram in the amygdala (Tovote et al., 2015). In parallel to the work in rodents, circuit and behavior analysis in invertebrates such as *Aplysia* (Kandel, 2001a)*, Drosophila* and *Caenorhabditis elegans* also reveal

specific circuit maps for various forms of memory and studies in these organisms provided some of the key insights to the cellular and molecular basis of memory engram (Ardiel and Rankin, 2010; McGuire et al., 2005).

1.2.2 Memory engram: cellular mechanism

Lashley's idea that learning builds an associative connection between neurons to form memory indeed can be dated back to the Spanish neuroanatomist Santiago Ramon y Cajal (1852-1934), who proposed that memories can be formed by strengthening the connections between neurons to improve the effectiveness of their communication (Cajal, 1894). Influenced by this, student of Lashley's, Donald Hebb (1904-1985), further conceptualized this theory into a simplified model of two neurons: "*When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased*" (Hebb, 1949). The Hebbian theory of memory was later being experimentally tested as the discovery of long-term potentiation (LTP): Timothy Bliss and Terje Lømo made an unexpected observation that the response of postsynaptic cell to the single-pulse stimuli of the presynaptic cell is enhanced following a high-frequency train of stimuli to the presynaptic cell, and this enhanced synaptic efficacy could last for hours (Bliss and Lømo, 1973). Through years of intensive research, LTP has become one of the cellular substrates for memory based on the following critical observations:

1. LTP can be observed following the inhibitory avoidance or spatial memory task in the hippocampus CA1 neurons or basolateral amygdala following auditory-fear conditioning and the effect could last for hours (Whitlock et al., 2006a). These and other studies suggest LTP is not an artifact of manipulated stimulation and natural experience can induce LTP.

2. Pharmacological or genetic inhibition of LTP blocks memory acquisition (McHugh et al., 1996; Morris et al., 1986). This suggests that LTP is necessary for memory to encode.
3. Recent advances in optogenetics (Boyden et al., 2005) allow to induce LTP optically in free moving mice (Zhang et al., 2008; Zhang and Oertner, 2007) and test the sufficiency of LTP in memory in temporal and spatial dependent manner. In one of the established memory paradigm, the auditory cued-fear conditioning, memory is the association of a tone and foot shock. Remarkably, substitution of the auditory input with optically generated LTP by stimulating channelrhodopsin-2 (ChR2) in the amygdala is able to encode and retrieve the fear memory (Nabavi et al., 2014a), suggesting LTP is sufficient for the two processes.

As the relationship between LTP and memory is interrogated, the molecular mechanisms underlying LTP has also begun to emerge. Because LTP changes the communication between neurons, the focus was first drawn to neurotransmitter and neurotransmitter receptor interaction. The N-methyl-D-aspartate (NMDA) receptor dependent LTP provided the first comprehensive picture of how LTP is initiated and sustained. Pre-synaptically released glutamate binds to postsynaptic NMDA receptors. Coincident activation and resulting depolarization of the post synaptic neuron removes the bound Mg^{2+} from the NMDA receptor. The removal of Mg^{2+} and binding of glutamate allows influx of calcium through NMDA receptors and activation of calcium/calmodulin-dependent protein kinase (CaMKII). The activated CaMKII phosphorylates a number of postsynaptic protein, including AMPA receptors, a second glutamate receptor. Phosphorylation of AMPA receptor changes the composition and abundance of AMPA receptors in the membrane and thereby fixing the increase in synaptic response. While the glutamate receptor represents just one of the many molecular cascades underlying LTP and memory, it points to the

importance of LTP as a critical knot connecting the memory engram from circuit to molecular level.

Although network and cellular logic of memory encoding and retrieval is becoming clear, the underlying molecular mechanisms remain unclear. This is because that most molecular studies lack temporal-spatial resolution to ask whether the molecular activity marks a persistent change in the engram cells or how does it react to retrieval of a memory, and how it connects encoding and retrieval. In the following section I have discussed the molecular pathways that have been implicated in LTP and memory.

1.3 Memory engram on the biochemical level

The existence of biochemical switches that can undergo persistence change in activity in response to a transient experience and creates a memory "engram" has been postulated for a long time. However, experimental evidence of the existence of such biochemical switches and more importantly what such switches are made of remain virtually unknown. As discussed above that three types of evidence should be considered in testing the biochemical engram: 1) must produce a persistent neurobiological change of a type that can account for the behavioral manifestation of memory; 2) must be utilized during memory retrieval; and 3) must link the processes of memory encoding and retrieval. Unlike the circuit engram that has endurable cellular material, biomolecules have short lives therefore a biochemical engram must be immune to turnover of individual molecules. I have discussed below the major biochemical pathways that have already been implicated in various aspects of memory and indicated whether they do or do not fulfill the criteria.

1.3.1 The cAMP pathway

The interest in the molecular component of memory engram started with the observation that applying monoamines (histamine and norepinephrine) or electrical pulses to the rabbit or guinea pig brain slices dramatically increases the content of adenosine 3',5'-phosphate (cyclic 3',5'-AMP, or cAMP) (KAKIUCHI and RALL, 1968a, b; Kakiuchi et al., 1969; McAfee et al., 1971). This suggested a molecular change in the neurons in response to stimuli. It was further shown in the gill-withdrawal reflex of the sea snail *Aplysia* (Castellucci and Kandel, 1976), elevated presynaptic cAMP induced by serotonin temporarily strengthened the sensory-motor neuron connection and facilitated the excitatory post-synaptic potential (Brunelli et al., 1976; Cedar et al., 1972; Cedar and Schwartz, 1972). Further studies in *Aplysia* and *Drosophila* Pavlovian conditioning (Quinn et al., 1974; Walters and Byrne, 1983) converged on the hypothesis that adenylate cyclase, which produces cAMP, serves as an intersection of inputs from conditioning stimuli and unconditioning stimuli (Abrams and Kandel, 1988; Byers et al., 1981; Eliot et al., 1989; Hawkins et al., 1983; Livingstone et al., 1984b; Uzzan and Dudai, 1982). The discovery of cAMP, cAMP-dependent protein kinases (Walsh et al., 1968) and downstream phosphorylation cascade established the role of cAMP as a cellular currency transforming extracellular stimuli to intracellular response, regulating neurotransmitter biosynthesis, post-synaptic response, and cytoskeleton dynamics (Castellucci et al., 1982; Castellucci et al., 1980; Greengard, 1978; Klein and Kandel, 1980).

1.3.2 Protein kinases

Phosphorylation is one of the most prevalent posttranslational modifications that switches protein activity in temporal and spatial manner. Elevated cAMP transduces the external signal to cAMP-dependent protein kinase or protein kinase A (PKA), one of the most studied protein kinases because of its wide function in multiple physiological systems. Its function in memory was

first identified in an invertebrate system. Mutations of genes encoding both catalytic and regulatory subunits of PKA (DC0 and PKA-R1) affect learning in *Drosophila* (Goodwin et al., 1997; Skoulakis et al., 1993). And enhanced PKA activity was observed in response to training using a FRET-based indicator (Gervasi et al., 2010). Mammals have multiple isoforms of PKA subunits, rendering it difficult to assign a specific role of PKA to memory by conventional knock out technique (Bernabeu et al., 1997a). However, expression of dominant negative PKA in mice inhibits L-LTP and long-term memory (Abel et al., 1997). Likewise pharmacological inhibition of PKA post-training inhibits memory consolidation however this effect was only restricted between 0-6h post-training (Bernabeu et al., 1997b). This result suggested PKA is required during early phase of memory consolidation while not the stable maintenance of memory for a long-term.

The framework that a protein kinase can maintain sustained activity insensitive to molecular turnover was raised in 1980s by John Lisman and others (Lisman, 1985). In this proposed theory, autophosphorylation of the kinase is the key step (Lisman, 1985). CamKII, because of its autophosphorylation activity (Barria et al., 1997) and activation in response to calcium influx mediated by NMDA receptor (Strack et al., 1997), became one such candidate. However, although autophosphorylation of αCamKII (T286) was observed for at least 8h post training (Barria et al., 1997), phosphorylation of its substrates was transient after LTP induction (Lengyel et al., 2004), suggesting an inhibitory mechanism of the kinase activity. Mutation that blocks autophosphorylation of αCamKII (T286A) showed impairment in LTP induction and learning (Giese et al., 1998). However, post-training pharmacologically interference with αCamKII activity did not impair memory storage or retrieval (Buard et al., 2010). Taken together, these observation suggested that CamKII is required for encoding of memory, however its activity is not required for persistence or retrieval of memory.

Another kinase pathway that is activated by activated NMDA receptor is the CaM kinase cascade, which eventually activates transcription through CREB. CamKIV is one of the critical players in this cascade that upon phosphorylation by CamKK translocates into the nucleus and phosphorylates and activates CREB (Bito et al., 1996). Mice expressing a dominant negative form of CaMKIV or CaMKIV deletion show impairment in late phase LTP and long-term memory, while early LTP and learning is not affected, suggesting unlike CaMKII, CaMKIV is important for long-term memory consolidation (Kang et al., 2001; Wei et al., 2002). Despite of these interesting observations, the dynamics of CaMKIV activity after encoding of a memory is not characterized. Future work should be focused on the persistence of CaMKIV activity post-training and whether it is required for retrieval of memory. These can be achieved by tools that allow temporally and spatially interfering the protein activity.

A brain-specific form of atypical PKC, PKMξ, a constitutively active kinase, is another potential candidate (Hernandez et al., 2003). Injection of a PKMξ inhibitory peptide (Hattori et al.) days after training erases a consolidated memory (Pastalkova et al., 2006). However, neither LTP or long-term memory was affected in PKMξ knock-out mice (Lee et al., 2013; Volk et al., 2013) suggesting either a compensatory effect in the absence of PKMξ (Tsokas et al., 2016) or ZIP targets other proteins (Lee et al., 2013; Volk et al., 2013) that might affect storage of memory. Despite the controversies, persistent increase of PKMξ was observed even after 1 month post-training (Hsieh et al., 2017). Development of genetic tools to modulate active PKMξ spatially and temporally may address its role in memory encoding, storage and retrieval.

cAMP level and protein kinase activity often show an immediate response and effect on synaptic plasticity, and most of them interfere with the animals' ability to learn. However, the molecular mechanisms that sustain a memory is not clear from these studies since no memory is

formed if the animal fails to learn. The first insight to the molecular transition from short-term to long-term memory was obtained from the vertebrate study, in which application of protein synthesis inhibitor blocked formation of long-term but not short-term memory (Berman et al., 1978; Davis and Squire, 1984; Eichenbaum et al., 1976). The systematic study of molecular transition from short-term to long-term memory is further carried out in the aforementioned sensory-motor neuron culture of *Aplysia* gill-withdrawal reflex, in which stimulation with quantitatively different serotonin produced different types of synaptic facilitation (Montarolo et al., 1986): one application of serotonin produces transcription and translation independent short-term facilitation, while five application of serotonin produces transcription and translation-dependent long-term synaptic facilitation, suggesting new gene products are necessary to establish long-term memory.

How are the cAMP pathway and the protein kinases cascade connected to make new gene products? The identification of cAMP-responsive DNA element (CRE) (Comb et al., 1986; Montminy et al., 1986) and the CRE-binding protein (CREB) (Montminy and Bilezikjian, 1987; Yamamoto et al., 1988), a transcription factor phosphorylated and activated by PKA (Gonzalez and Montminy, 1989) in mammalian cells, paved the way to connect the cAMP signaling to transcriptional activation. Other activity dependent protein kinases such as CaM kinase and MAPK kinase cascade also lead to activation of CREB (Giese and Mizuno, 2013). The same pathway is shown with *Aplysia* CREB-1 in sensorimotor neuron culture as a mechanism in long-term facilitation (Dash et al., 1990). CREB-1induces the expression of several immediate early genes essential for long-term facilitation, such as another transcription factor C/EBP to induce a second wave of gene expression (Alberini et al., 1994). This CREB-dependent long-term memory is conserved in many organisms (Bourtchuladze et al., 1994; Frank and Greenberg, 1994; Guzowski

and McGaugh, 1997; Lamprecht et al., 1997; Silva et al., 1998; Tubon et al., 2013; Yin et al., 1994a). Therefore, cAMP and protein kinase signaling bridge short-term and long-term memory, establishing a central pathway for the molecular memory.

1.3.3 Transcription factors

CREB, as a transcription factor integrating signals from cAMP pathway and protein kinases, shifts the focus of molecular cascade of memory into the transcription event in the nucleus. Although much of the focus is on the effect of translational inhibition on memory consolidation, in the 1960s, drugs that inhibit transcription also showed memory impairment across species and in different training context (Da Silva et al., 2008; Eugenia Pedreira et al., 1996; Frey et al., 1996; Neale et al., 1973; THUT and LINDELL, 1974). However, the unspecific toxicity of these drugs limits their use in research (Wetzel et al., 1976). In the meantime, the general picture of transcription machinery begun to emerge, as well as the tools to target specific transcription regulators, which benefited the study of transcription and memory consolidation.

1.3.3.1 CREB and C/EBP

As discussed above, multiple studies suggest an evolutionary conserved central role of CREB in memory consolidation. Here I discuss whether CREB meets the 3 criteria as an engram molecule, or physical substrate of memory:

1. Is acquisition of memory and activity of CREB linked? The state of ser-133 phosphorylated CREB (pCREB) is considered to be a marker of activated CREB. In *Aplysia* neuron culture, pCREB1 shows an interesting biphasic profile, with the first wave spanning 10-40min after exposure to 5-HT, and the second wave arising 1h after extended 5-HT exposure and persisting for 12h after removal of 5-HT (Bartsch et al.,

1998). Studies in rat dentate gyrus also suggests a biphasic activation of CREB (Schulz et al., 1999) following LTP induction. Similarly, behavioral training such as inhibitory avoidance task, induces a biphasic activation of pCREB in the hippocampus: the first wave arises immediately after training and drops to baseline at 30min, and the second wave peaks between 3-9 h post training (Bernabeu et al., 1997b; Stanciu et al., 2001) and in some cases sustains 20h after training (Taubenfeld et al., 2001a). These observations suggest CREB activation coincides with acquisition of memory.

2. Is the signal transduced to CREB persistent over time in the absence of stimulation?

   The biphasic profile of pCREB after training could potentially reveal a cyclic molecular program that persists overtime after training. Indeed, experiment using RNA polymerase II inhibitor also reveals two transcription sensitive periods of memory consolidation process (Igaz et al., 2002; Quevedo et al., 1999), suggesting persistent need of transcription. Later in *Aplysia* a positive-feedback loop mechanism is proposed for self-sustained CREB1 activation, through a cAMP-response element residing in the CREB1 promoter (Liu et al., 2008; Mohamed et al., 2005), a possible explanation for the biphasic of CREB activation. However, CREB tends to incorporate multi-dimensional information in the scenario of whole animal. For example, oscillation of pCREB is observed through circadian cycle in *Drosophila* (Tanenhaus et al., 2012; Zhang et al., 2013), in the REM sleep of mammal (Luo et al., 2013), stress and growth stimulation (Lonze and Ginty, 2002). Therefore, although a self-regulated persistent change of CREB may exist post-training, as it incorporates various physiological information, its function in persistent storage of memory is difficult to resolve.

3. Is CREB necessary or sufficient to recall a memory?

It's becoming clear that CREB is both necessary and sufficient for initial memory consolidation. Its role in memory recall is not fully understood. In rat conditioned taste aversion task, oligodeoxynucleotides antisense against CREB is shown to affect specifically long-term but not short-term memory. The antisense oligo also does not interfere with long-term memory retrieval when applied right before memory test (Lamprecht et al., 1997). This suggests CREB is not necessary for memory to recall. However, the retrieval process does elevate the level of pCREB (Hall et al., 2001b), which is critical for the reconsolidation and extinction process of memory after retrieval (Kida et al., 2002; Tronson et al., 2012).

To conclude, CREB can transduce the external information by engaging to an active form that persist up to 24h, but is not required for retrieval of memory in the long-term. Moreover, the CREB transcription factor family has complicated genomic structure leading to alternative splicing that generates transcription activator or repressor (Bartsch et al., 1998; Mayr and Montminy, 2001; Tubon et al., 2013). These features suggest CREB is component of memory engram but does not fully represent it.

The interesting aspect of transcriptional regulation on memory consolidation is that it often manifests in two or maybe multiple waves, either reusing the same sets of transcription activator (positive feedback loop of CREB) or induction of new cascades of transcription activators. For example the CCAAT enhancer-binding element (C/EBP) transcription factor family, a target of CREB, is detectable ~9h after training, in the same sets of neurons that show pCREB signal at early time point (Taubenfeld et al., 2001b). This elevated expression although persists up to 48h, like CREB, C/EBP is although necessary for memory formation but not necessary for memory retrieval (Taubenfeld et al., 2001a).

1.3.3.2 Immediate early genes

Besides C/EBP, a myriad of transcription factors, termed as immediate early genes (IEGs), can also be detected upon stimulation of neurons or neuroblastomas such as PC12 cells. The activator protein 1 (AP-1), composed of Fos, Jun and ATF heterodimers, is one of the earliest IEGs being discovered. c-fos is one of the earliest IEGs identified in cells exposed to growth factors (Greenberg and Ziff, 1984). Together with c-fos, other IEG transcription factors, zif268, c-jun, jun-B are found to be rapidly induced in cells receiving neuronal growth factors.

The molecular pathway pinned down in PC12 cells paved the way for *in vivo* analysis of IEGs in the nervous system. Various brain stimulations including pharmacologically induced seizures, electrical stimulation, neurotransmitter receptor agonist, light, and surgical lesions robustly induce IEG expression (Morgan and Curran, 1991). Unlike the consistent response induced by seizures, IEGs that respond to long-term potentiation (Nabavi et al., 2014b; Whitlock et al., 2006b), are more variable between different IEGs and across brain regions. For example in the hippocampal dentate gyrus, frequency that produces long-term potentiation does not consistently lead to c-fos induction (Cole et al., 1989; Douglas et al., 1988); in contrast, zif/268 expression correlates better with LTP (Cole et al., 1989). Although the induction of zif268 is transient (decay within 3h), it is tightly associated with duration of LTP compared to other IEGs (c-fos, c-jun and junB) examined thus far (Abraham et al., 1991; Richardson et al., 1992). Consistently, in zif268 mutant mice early phase LTP is normal but the late phase of LTP is defective (Jones et al., 2001).

In the natural behavior settings, the expression of IEGs is even more controversial. In contextual fear conditioning, zif/268 shows enhanced labelling in the amygdala neurons specifically in conditions that produces memory, while c-fos positive neurons appear in animal

with or without memory (Rosen et al., 1998). In another series of studies, zif/268 expression in the amygdala is associated with retrieval but not acquisition of contextual fear conditioning (Hall et al., 2000), (Hall et al., 2001a). In the conditioned taste aversion task, however, c-fos is critical for memory retention in the brainstem nuclei while zif/268 is not necessary (Yasoshima et al., 2006). Surprisingly, although c-fos plays central roles in neuronal activity, the c-fos genomic deletion mice did not show memory impairment (Wang et al., 1992), suggesting other signaling pathway compensating for its function (Yasoshima et al., 2006). In contrast, long-term memory retention in both water maze and conditioned taste aversion was impaired in zif268 deletion mice (Jones et al., 2001).

Nevertheless, c-fos was induced in both the primary neurons receiving stimulation and the secondary neurons activated by the primary neuron, suggesting c-fos labels an ensemble of activated neurons (Sagar et al., 1988). Although inhibition of c-fos before or during training interferes with long-term memory consolidation in various tasks (Grimm et al., 1997; Lamprecht and Dudai, 1996; Morrow et al., 1999), its wide expression indicate a general function in the nervous system as oppose to a specific function in memory.

The general difference between IEG transcription factors and CREB is that the IEGs are regulated at the transcription level, while CREB is modulated by post-translational modification. However, the rapid and transient induction of IEGs, although correlates with LTP and consolidation of long-term memory, does not persist the entire duration of a memory, therefore is unlikely to serve as a biochemical engram for memory by themselves. However, taking advantages of their transient induction during memory encoding, they have been used to label neurons that are recruited during memory encoding and activation of those neurons are sufficient to recall the memory (Tonegawa et al., 2015).

1.3.4 Epigenetic labeling

Transcription factors, including CREB and IEGs, also bring chromosome modifiers to alter the genome architecture and hence gene expression and neuronal function. Since the epigenetic mechanisms, such as DNA methylation or histone acetylation, are known to persistently alter gene expression, could the alteration on a chromosome last long enough to serve as a cellular marker for long-term memory?

1.3.4.1 Histone modification

The CREB binding protein (CBP) is a well-studied histone modifier critical for memory consolidation. CBP has two functions: first, the CREB/CBP complex can serve as a platform for recruiting other components of the transcription machinery and second, it contains a histone acetyltransferase domain. It was discovered in *Aplysia* sensory motor neuron culture that CREB1/CBP acetylase complex was recruited to the promoter region of C/EBP and correspondingly increase H3 and H4 acetylation. However, just as the transient induction of C/EBP mRNA, acetylation decreases within 2h post 5-HT stimulation, with replacement of CREB1/CBP complex with CREB2/HDAC5 deacetylase complex to the C/EBP promoter (Guan et al., 2002). Consistently, in rats training with contextual fear conditioning, H3 acetylation is increased 1h after training but the elevation dose not last to 24h (Levenson et al., 2004). Overexpression of a dominant-negative form of CBP that specifically blocks its histone acetyltransferase activity inhibits induction of c-fos and memory consolidation while leaving short-term memory intact, in both visual-paired comparison task and water maze (Korzus et al., 2004). Conversely, several HDACs have been shown to have negative effect on LTP and memory, although to varying degree. For example overexpression of HDAC2 impairs while knockout enhances memory consolidation, through regulating histone acetylation on the promoter of various memory related genes (Guan et

al., 2009). HDAC3 is also a negative regulator of memory and HDAC3 deficient mice show memory enhancement (McQuown et al., 2011). In object recognition/location memory task, post-training injection of an HDAC3 inhibitor RGFP136 prolongs memory to 7 days, while the memory in vehicle control can only last for 24h (McQuown et al., 2011). Similarly, injection of a general HDAC inhibitor trichostatin A (TSA) before or right after training enhances CREB/CBP dependent memory consolidation in a brain region-task specific manner, while injection of TSA during memory retrieval does not have an effect (Vecsey et al., 2007). In conclusion, these results suggest active histone acetylation and deacetylation happen during or right after training, possibly through regulation on the promoter of several immediate early genes. However, there are still unanswered questions regarding whether histone acetylation could be a biochemical engram:

1. Enhanced but transient histone acetylation is observed right after external experience on specific promoter region (Guan et al., 2002; Levenson et al., 2004). However, the persistency of histone acetylation is unclear. Although it disappears on the C/EBP promoter, the acetylation code could be transduced to other downstream promoter regions of memory related genes yet examined.
2. In most cases, the effect of HDAC inhibitor on memory enhancement is time limited – must be applied right after training (McQuown et al., 2011; Vecsey et al., 2007); enhanced acetylation does not help in memory retrieval (Vecsey et al., 2007). However, in the mice model of massive neuron loss, chronic injection of another HDAC inhibitor, sodium butyrate (SB), before test leads to increased histone acetylation and the ability to retrieve memory (Fischer et al., 2007), suggesting histone acetylation is sufficient to reinstate the memory accessibility in this specific model. Whether histone acetylation could store and retrieve a memory in healthy animal is waiting for more detailed study. One caveat of the

pharmacological inhibitors is their specificity with respect to substrate, cell type and time. Development of genetic tools to target specific histone acetylase and deacetylase in time and space dependent manner should help to enhance the resolution of histone modification in memory.

3. The fact that memory can be enhanced by HDAC inhibitors through enhancement of histone acetylation, is a nice demonstration of the sufficiency of histone acetylation as a memory substrate (McQuown et al., 2011; Vecsey et al., 2007). However, since it's an indirect way, the consequences of HDAC inhibition calls for careful examination. For example, it's not clear how after application of HDAC inhibitors the acetylation code persist: is it still on the same regions or expands/migrates to other genomic regions? It's also not clear how training regulates HDAC2/3 activity and why the system needs a constraint on training induced gene expression. One possibility is to trigger forgetting and memory extinction, and regulate strength of memory.

In summary, histone acetylation is a promising molecular engram. However, to determine such memory trace, detailed analysis like histone modification profiling on different genomic regions across different time post-training might reveal the whole picture.

1.3.4.2 DNA methylation

Besides histone modification, DNA methylation is also involved in regulation of gene expression upon stimuli. The idea of DNA methylation as a memory trace that lasts for a lifetime is compelling, given that methylation of DNA can change gene expression permanently and even transgenerationally (Santos et al., 2005). DNA methylation on memory inhibiting gene PP1 and demethylation on a memory enhancing gene reelin have been reported to happen cooperatively in the hippocampus CA1 neurons 1h after contextual fear conditioning. However, the alteration in

methylation on both sites disappear 24h later (Miller and Sweatt, 2007). Intra CA1 infusion of DNA methyltransferases inhibitor before and right after training blocks memory consolidation while does not interfere with memory retrieval when applied before test (Miller and Sweatt, 2007). This suggests that transcriptional inhibition of certain genes (memory repressors) are necessary for memory consolidation. And unlike the stable DNA methylation in the developmental process, DNA methylation in response to behavioral training is rapid and reversible. Fascinatingly, recent memory in the mammalian brain specifically undergoes system consolidation into remote memory that can last months. In the contextual fear conditioning per se, the memory trace is transferred from hippocampus to prefrontal cortex under system consolidation (Dudai, 2004). And it is found that DNA methylation on the memory repressor gene calcineurin (a phosphatase) (Malleret et al., 2001) in the prefrontal cortex is enhanced 1d later and persists for at least 30d (Miller et al., 2010), a time frame that consistent with system consolidation (Frankland et al., 2004). Moreover, pharmacological inhibition of DNA methylation in the prefrontal cortex 30d after conditioning specifically affect memory retrieval at this point but if applied in the prefrontal cortex 24h after training, it does not affect memory afterwards (Miller et al., 2010). This strongly suggests DNA methylation in the prefrontal cortex is necessary for memory to be retrieved. Therefore, DNA methylation on the gene region of calcineurin might be a good candidate to trace remote memory in the prefrontal cortex. However, it might not be a generalized mechanism, given that hippocampus also stores protein synthesis dependent memory for ~3d while DNA methylation is transient (within 1h). DNA methylation is not well conserved in invertebrate organisms; it may be a newly evolved mechanism for long-lived animals to preserve remote memory.

1.3.5 Translational machinery

1.3.5.1 Early evidence of localized translation

IEGs, in addition to transcription factors, also contain a second category, the "effectors". Arg3.1/Arc is one such gene that is induced in the dentate gyrus following LTP and shows a similar expression profile as that of zif268 (Link et al., 1995; Lyford et al., 1995). Because of its interaction with F-actin, it is named as an activity-regulated cytoskeleton-associated protein (Arc) (Lyford et al., 1995). Later it was shown that Arc mRNA can be localized selectively to the activated dendrites in the dentate gyrus (Steward et al., 1998; Wallace et al., 1998), and this localization is dependent on NMDA receptor activation (Steward and Worley, 2001). Similarly, detection of increased αCaMKII protein in the dendrites was reported, 5min after tetanus in dendrites 100-200um away from the soma (Ouyang et al., 1999). The increased dendritic proteins could come from two sources: proteins that synthesized in the cell body then transported to the synapses or proteins that are directly translated from the synaptically localized mRNA. As proteins in the neurons are transported at a rate of 1.4-2.8 um/min (Brady and Lasek, 1982), this rapid increase of CaMKII protein cannot come from the cell body. These observations point to a new direction that translation of localized mRNA at activated synapses may serve to maintain late phase LTP and long-term memory. Interestingly, in the spatial water task, when assayed 30min post training, the level of Arc mRNA in hippocampus shows significant linear correlation with the acquired memory on the individual animal level (Guzowski et al., 2001), suggesting the mRNA of Arc could serve as biochemical marker for acquired memory. However, the induced Arc mRNA disappear within 6h post-training, indicating it's unlikely to be a biochemical trace for memory in the long-term.

Focus then shifts to the translational control of target mRNA locally in response to neuronal stimuli. In the early 1980s, polyribosomes were found in the dendritic region in the dentate gyrus, especially during re-innervation, suggesting the possibility of local protein synthesis (Steward and Fass, 1983; Steward and Levy, 1982). Direct evidence of dendritic protein synthesis came from

the observation that rapid and three-fold increase in ^{3}H-leucine labelled peptide in dendrites upon stimulation (Feig and Lipton, 1993). Consistent with these observations, protein synthesis in the dendritic transections is preserved in response to stimulation in both mammalian hippocampal post-synaptic dendrites (Aakalu et al., 2001) and *Aplysia* pre-synapse of sensory neurons (Martin et al., 1997), confirming that the synapses contain all the necessary components (mRNA and translational machinery) for new protein synthesis.

Can components of translational machinery be a reliable biochemical engram for long-term memory? Translational control in response to LTP could be regulated at two points, through a translation initiation/elongation complex that has general affect, or through gene specific mRNA binding proteins (Pfeiffer and Huber, 2006).

1.3.5.2 General protein synthesis

The fact that application of protein synthesis inhibitor inhibits the consolidation of long-term memory (Kandel, 2001b) suggests that interference of general protein synthesis could perturb memory consolidation.

Translation of new proteins is regulated at three steps: initiation, elongation and termination. Initiation is the rate limiting step and regulation of several initiation factors have been implicated in memory process. Phosphorylation of the alpha subunit of eIF2 (eIF2α) is one of the hall marks of translational activity: elevated p-eIF2α blocks translational initiation, thereby new protein synthesis, whereas reduced p-eIF2α associates with enhanced translation (Pavitt et al., 1998). Stimuli that induce synaptic plasticity reduce p-eIF2α (Costa-Mattioli et al., 2005; Costa-Mattioli et al.; Takei et al., 2001). Consistently, mutation that blocks eIF2α phosphorylation or small molecule that inhibits eIF2α kinase PERK enhances L-LTP, spatial memory, contextual and

auditory fear conditioning and taste aversion memory; while peptide that prevents eIF2α dephosphorylation blocks L-LTP and consolidation of long-term memory (Costa-Mattioli et al.; Sidrauski et al., 2013). Remarkably, suppression of PERK can even alleviate defects of synaptic plasticity and spatial memory in the Alzheimer's disease model mice (Ma et al., 2013). Worth mentioning, p-eIF2α signal is tightly associated with stress response, in which cells suspend translation in unfavorable conditions. A subset of mRNAs that contain upstream open reading frames (uORFs) are upregulated upon increased p-eIF2α during stress conditions. Among them, ATF4, a repressor of CREB, links general translation inhibition to CREB mediated transcription (Costa-Mattioli et al.). Since activation of neurons can also be considered as stress to the neurons, it remains to be determined the interaction between stress response and learning and memory.

The eIF4F translation initiation complex is also the target of activated translation in response to neuronal stimuli. eIF4F complex is composed of the DEAD-box RNA helicase eIF4A, the cap-binding protein eIF4E and the scaffold protein eIF4G. Regulation of translation in condition of neuronal activity is primarily through two pathways: phosphorylation of eIF4E, or phosphorylation of eIF4E-binding proteins (4E-BPs). Phosphorylation of eIF4E is induced by both protein synthesis dependent induction of LTP and LTD through a MAPK-dependent pathway (Banko et al., 2004; Kelleher Iii et al., 2004). Expression of dominant negative form of MAPK inhibits 24h memory, L-LTP and cap-dependent translation. However, this type of regulation exists neuron-wide therefore cannot explain the localized translation (Kelleher Iii et al., 2004).

In contrast to phosphorylation of eIF4E, translation control through mTOR-dependent phosphorylation of 4E-BPs may have selective targets. Unphosphorylated 4E-BP competes with eIF4G to bind eIF4E thereby inhibit cap-dependent translation. L-LTP and LTD simulate activity of mTOR to phosphorylate downstream 4E-BPs and leads to translation of a number of mRNAs

that contain a 5' terminal oligopyrimidine tract (5'TOP) (Antion et al., 2008; Carroll et al., 2006; Carroll et al., 2004; Hou and Klann, 2004). Importantly, the 5'TOP containing mRNAs are enriched with components of translational machinery such as ribosomal proteins and elongation factors (Meyuhas and Kahan, 2015). How these mRNA get translated remain elusive but could provide possible mechanism of the activity-dependent translation of specific target.

Besides cap-dependent translation, ribosomes can also be recruited to a highly structured 5' UTR region called internal ribosome entry site (IRES). Although first identified in viral RNA, IRES is also present in a number of eukaryotes mRNA encoding proteins needed during stress response (Holcik and Sonenberg, 2005) as well as dendritically localized neuronal mRNAs (Pinkstaff et al., 2001). However, only one observation in *Aplysia* suggests activity triggers a switch from cap-dependent to cap-independent translation via eIF4E dephosphorylation (Dyer et al., 2003). The IRES mediated activity-dependent translation is still a hypothesis.

Traditionally, translation initiation is thought to be the rate-limiting step in activity-dependent protein synthesis. Cumulative evidence suggest polypeptide elongation step can also be a target of regulation (Richter and Coller, 2015). This is of specific interest in highly polarized neurons since it has long been observed that translocation of mRNA in neurons is through discrete granules known as RNA granules composed of polyA mRNA, polyribosomes and elongation factors but devoid of translation initiation complex (Knowles et al., 1996). Interestingly, these RNA granules show a heavily packed assembly and translationally inert, however, could reorganize into translational active polyribosomes upon stimulation (Krichevsky and Kosik, 2001). This process is directed by phosphorylation of elongation factor eEF2 (Scheetz et al., 2000; Sutton et al., 2007). One interesting but not fully understood aspect of eEF2 regulation is that phosphorylation of eEF2 generally abort translation however could specifically activate translation

of activity dependent synaptic proteins such as Arc (Park et al., 2008) and CaMKII (Scheetz et al., 2000). This unique property could amplify the difference in protein abundance between activity and synaptic plasticity-dependent gene products and activity-independent genes. However, no mechanism has been proposed yet.

Could components of translation complex serve as molecular engram for memory? It is no doubt that new protein synthesis is required for establishment of long-lasting memory. This is likely due to translation of specific but not all mRNAs. However, the translation complex is involved in general protein synthesis which is required for normal physiological function. Monitoring the translation complex activity on specific target spanning the life cycle of a memory is technical challenging.

1.3.5.3 mRNA binding proteins

Cis-regulatory information on the mRNA sequence combined with the recognition specificity of the mRNA binding proteins can potentially determine the translation of target mRNA in activated synapses (Shi and Barna, 2015). In neurons, neuronal granules are enriched with translationally stalled mRNA as well as a large number of RNA-binding proteins. Interestingly, most of these RNA-binding proteins hold target mRNA in a translation repressive state, protecting mRNA from degradation, guiding mRNA transportation and localization, and sensing activity of the neurons to release mRNA for translation (Thomas et al., 2014). Here by comparing with some mRNA binding proteins that have been implicated in activity dependent translation in the nervous system, I extracted the unique feature of the prion-like CPEB proteins.

FMRP

The Fragile X Mental Retardation Protein (FMRP) is perhaps the most studied mRNA binding protein due to its direct link to Fragile X syndrome, which is manifested with neurodevelopmental delay, cognitive dysfunction, hyperactivity and autistic-like behavior (Santos et al., 2014). Mutations identified in human show CGG triplet expansion in the 5' UTR of the gene which leads to excessive DNA methylation and silencing of the gene (Pieretti et al., 1991; Verheij et al., 1993). Mouse and fly model with fmr1 knock-out were subsequently generated and partially represented the human Fragile X syndrome (Kooy et al., 1996; Zhang et al., 2001). Research on the mouse model did not reach consistency especially on the function in learning and memory. For example, Fmr1 KO mouse did not show impairment in learning, memory consolidation and retrieval in Morris water maze (Kooy et al., 1996) however show reduced memory retention in Barnes maze (Yan et al., 2004). Investigation on the associative learning also ended with mixed results (Dölen et al., 2007; Michalon et al., 2012; Yuskaitis et al., 2010). Because FMRP is involved in development of nervous system, emotion, social communication and circadian rhythm (Santos et al., 2014), which all pinch on the knock-out mice model, making it difficult to address whether FMRP has specific function in memory. The underlying molecular mechanism is not well-understood either, with some report suggested it as a general translation inhibitor (Dölen et al., 2007) while other suggested a dual-function (Brown et al., 2001; Napoli et al., 2008). Tools to dynamically modify the FMRP activity are desired to answer its roles in memory.

Hu

The *Drosophila* Elav protein is an mRNA binding protein essential for nervous system development and function (Campos et al., 1985). The mammalian homolog HuD has been shown to be up-regulated in hippocampal neurons after contextual (Bolognani et al., 2004) or spatial learning (Pascale et al., 2004). Consistently, it has been confirmed that HuD stimulates translation

of several target genes related to synaptic plasticity and memory (Tanner et al., 2008; Vanevski and Xu, 2015). As Hu is essential for development, knock-out animal may not be viable. However, overexpression of HuD interferes with memory acquisition and retention (Bolognani et al., 2007), suggesting a balance of translation has to be maintained for memory processing. It's unclear at this stage how HuD affect different stages of memory.

CPEB protein family

One of the mRNA binding proteins that shows specificity in binding and regulation of mRNAs that contribute to synaptic function is cytoplasmic polyadenylation element binding protein (CPEB). Almost all eukaryotic pre-mRNAs (except histone mRNAs) undergo co-transcriptionlly addition of nontemplated polyA tail following cleavage at 3' end, resulting in a stretch of 200-300 adenosine residues (Zhang et al., 2010). This process occurs in the nucleus and is dependent on the nuclear polyadenylation complex. The polyadenylated mRNA is escorted to the cytoplasm, where translation takes place. Although in most cases, elongated polyA tail correlates with increased translation and shortened polyA tail exposes the mRNA for degradation, in some cell types specifically oocyte and neurons there are exceptions. In most cells mRNA maintains an average half-life of 9h. While in oocyte and neurons, a number of mRNAs stay dormant in preparation for sequential activation signals. One of the best examples of such process is the maternal mRNA translated during oogenesis. In *Xenopus* for example, oogenesis could span for months with only maternal mRNAs and proteins, which means the maternal mRNAs need to be stabilized and protected before active translation. Shortening of polyA tail and shielding by other mRNA binding proteins are strategies to preserve these mRNAs for further use. The role of CPEB1 is best characterized to fit this requirement in maturation of *Xenopus* oocyte. During this process, maternal mRNA that contains CPE element in the 3'UTR are selectively bound and

regulated by CPEB1. CPEB1 associates both deadenylating enzyme polyadenylate-specific ribonuclease (PARN) as well as non-canonical polyA RNA polymerase germline development 2 (GLD2). In the absence of translation simulation, PARN shortens polyA tail. CPEB1 also recruits another translation inhibitor maskin which occupies eIF4E and prevents assembly of eIF4F translation initiation complex. In response to timely stimulation (progesterone, fertilization), Aurora A kinase phosphorylates CPEB1 thereby releases PARN and allows polyA elongation by GLD2. PolyA tail further recruits PABP and eIF4G to compete maskin for binding with eIF4E to trigger active translation (D'Ambrogio et al., 2013).

Studies of CPEB1 regulated translation in the nervous system suggests overlapping molecular complex shared by oocytes and post-synaptic dendrites. CPEB1 is present in both soma and dendritic area and bidirectionally regulates translation of dendritic mRNA, such as α-CamKII and NMDA receptor subunit NR2A, in an activity dependent manner (Huang et al., 2002; Udagawa et al., 2012; Wu et al., 1998). Conversely, α-CamKII also phosphorylates and activates CPEB1 (Atkins et al., 2004). Induction of L-LTP can induce prolonged phosphorylation of CPEB1 beyond 30min post stimulation and the maintenance of high level of phosphorylated CPEB1 is achieved by both activation of α-CamKII and inhibition of CPEB1 phosphatase PP1 (Atkins et al., 2005). Therefore, the maintenance of active CPEB1 mediated polyadenylation and translation is through the balance of upstream kinase and phosphatase. Alternatively, CPEB1 and α-CamKII could form a self-sustained feedback loop.

Can CPEB1 maintain active translation after stimulation and training? Although phosphorylated CPEB1 can be rapidly induced by neuron depolarization, the transient phase of this induction (rise and drop within 1 min) suggests activated CPEB1 cannot be self-maintained and is prone to be deactivated (Atkins et al., 2004). In spite of well understood molecular

mechanism of CPEB1 in localized translation, the necessity of it in LTP and memory is less understood. Curiously, only selective forms (single train of theta-burst) of LTP is affected in CPEB1 deficient mice (Alarcon et al., 2004) and the CPEB1 knock out mice does not show impairment in memory consolidation but impairment in memory extinction (Berger-Sweeney et al., 2006). This suggests two possibilities: either CPEB1 is not the critical molecule that sustains active translation for memory to maintain, or other molecular complexes could compensate in the absence of CPEB1. The fact that the existence of other CPEB-like (CPEB2-4) proteins in the nervous system (Theis et al., 2003) cannot explain the compensation for CPEB1, because CPEB2-4 does not recognize CPE but other RNA sequences that could engage complex secondary structures (Huang et al., 2006b). Likewise, most post-translational modification happens within 1h after LTP or memory training, phosphorylation of CPEB1 in this case may not be sustained beyond the post-translational modification time window. These results suggest CPEB1, although specifically regulates translation of mRNAs that are critical for memory consolidation, may not be a candidate as molecular memory engram.

The discovery of a Q/N rich prion-like domain reside in the N-terminus of other CPEB family member, namely ApCPEB in *Aplysia* (Si et al., 2003b), Orb2 in *Drosophila* (Keleman et al., 2007; Majumdar et al., 2012) and CPEB2-4 (Fioriti et al., 2015; Huang et al., 2006a) in mice, opens up an alternative regulation path independent of the canonical Aurora A kinase-CPEB polyadenylation pathway. The prion-like domain is necessary for the protein to adopt an SDS-resistant, ThT positive, insoluble oligomeric form upon stimulation and memory training, as well as maintenance of long-term facilitation in *Aplysia* (ApCPEB) (Miniaci et al., 2008; Si et al., 2010; Si et al., 2003a) and long-term memory in flies (Orb2) (Majumdar et al., 2012) and mice (CPEB3) (Fioriti et al., 2015). Interestingly, all CPEBs studied seem to have dual functions, switching

between translational activation and repression in response to cell signaling and post-translational modifications. Aurora kinase A phosphorylates CPEB1 and remodels the CPEB1 associated mRNP complex from a repressor to an activator. CPEB3 and homologs in *Aplysia* (ApCPEB) and *Drosophila* (Orb2) are, via the dynamic oligomerization of their prion-like domains, critical for the persistence of synaptic facilitation and maintenance and expression of long-term memory. Remarkably, work from *Drosophila* Orb2 suggests it forms distinct protein complexes in monomeric and oligomeric forms: monomeric Orb2 associates with a deadenylation complex and shortens the polyA tail of target mRNA, while oligomeric Orb2 associates with a polyadenylation complex to preserve the target mRNA's elongated polyA tail (Khan et al., 2015). In mammalian neurons, CPEB3's switch between oligomeric translational activation and monomeric translational repression is regulated by SUMOylation and ubiquitination. The aggregated CPEB-like proteins persist through different time scales in different models: in *Aplysia* and *Drosophila*, aggregated CPEB are still significantly higher 24h post stimulation while in mice CPEB3 aggregates can sustain to 24h with a reduced level compared to 1h post training. This suggests in higher eukaryotes, a stringent regulation might take place to prevent the overgrowth of aggregation. Nevertheless, the time scale is beyond post-translational modification that is induced within 1h after training. Another fundamental difference between the amyloid-like assembly of prion-like CPEB and RNP assembly of CPEB1 is the prion-like assembly is not dependent on RNA and does not colocalize with P body or RNA granules (Fioriti et al., 2015; Si et al., 2010). Of most low complexity sequence RNA binding proteins identified so far, aggregation usually functions to inhibit protein translation. Prion-like CPEB is a rare example whose aggregation is associated with active translation. This unique property allows it to maintain sustained active translation after transient stimulation and beyond post-translational modifications in the following ways:

1. The amyloid-like assembly unlike other RNP granules does not depend on target mRNA. In other words, the nucleation is not RNA based, instead, it is protein conformation based. Amyloid-core structure resides within the assembly not RNA, while the RNA locate in the outer layer of the assembly. As memory consolidation during different time windows might call for different mRNA substrates, this organized structure allows dynamic exchange of mRNA targets, not the core proteins.
2. Unlike other RNP granules which contain a variety of RNA binding proteins with low-complexity domain, amyloid-like assembly is usually very homogenous in the core proteins. This structure is much more stable than the heterogenous RNP granules in a sense that only proteins of the same kind can be specifically recruited. This may benefit the stable storage of a memory trace that can sustain physiological metabolism within the busy cellular milieu.
3. The stability of amyloid-like assembly does not mean the structure is rigid and does not refresh itself. Indeed, the specific homo-interaction ensures the refresh process takes place, reducing the risk of disassembly. This allows the memory to be modified but still keep the trace generated by previous experience.

1.4 Hypothesis and objectives

The circuit analysis of encoding, storage and retrieval of a memory has a long history and the recent advance in molecular biology and optogenetics further fill up the picture. Imaging of activated neurons and manipulation of neuronal activity in *Drosophila* (Davis, 2011) and in mice (Cowansage et al., 2014; Garner et al., 2012; Josselyn et al., 2015; Liu et al., 2012; Ramirez et al., 2013) has led to the identification of sets of neurons or "engram" cells that are recruited when

memories are formed and activation of these neurons are necessary and sufficient to retrieve a memory. However, the biochemical changes in the engram cells that allow storage and recall of memory remain unclear. To date, hundreds of molecules have been shown to affect memory: signaling molecules, transcription factors, epigenetic modifiers and translation regulator. However, very few of them by themselves fulfill the criteria of a molecular engram of memory.

The prion-like neuronal CPEB proteins act as a bi-stable switch. Our hypothesis is that experience converts it from a monomer into a self-sustaining aggregated state and creates an enduring biochemical alteration in specific neurons (and synapses), maintains memory over time and acts to retrieve memory. This model raises the following fundamental yet unanswered questions which are the objectives of my research: 1) where in the brain Orb2 activity is required to form a stable memory? 2) What aspects of memory are dependent on Orb2: encoding, storage or recall? 3) How does perturbation of Orb2 aggregation affect memory? 4) Where does Orb2 aggregation occur and can aggregated status of Orb2 inform about the strength of memory? My PhD studies have addressed these questions.

Chapter 2. Material and Methods

2.1 Generation of transgenic constructs and fly strains

Modified Orb2 genomic rescue construct: A 18,761 bp genomic fragment encompassing the Orb2 locus was cloned into a pattB vector to generate the pattB-Orb2 construct. The pattB-Orb2 construct was described in detail previously in Majumdar et al. (Majumdar et al., 2012). For this study the pattBOrb2 construct was further modified to generate the following constructs by counter selection BAC modification: 1) pattB-Orb2TevS216: the TEV protease recognition site ENLYFQG was inserted at the amino acid position 216 with respect to the Orb2A protein; 2) pattB-Orb2TEV-N: The N-terminal TEV fragment (1-118aa) was fused to the C-terminal of Orb2 with the linker sequence SRPGS; 3) pattB-Orb2TEV-C: The C-terminal TEV fragment (119-242aa) was fused to the C-terminal of Orb2 with the linker sequence SRPGS; 4) pattB-Orb2ΔATEV-N and pattB-Orb2ΔATEV-C: the first 8 amino acids specific to Orb2A protein, MYNKFVNF, were deleted from pattB-Orb2TEV-N and pattB-Orb2TEV-C constructs. All genomic rescue constructs were inserted at attP2 site in the 3rd chromosome and then recombined with Δorb2. These constructs fully rescue the lethality of the Orb2 null mutant.

The pUAST-GFPdark construct: The quenching peptide (Nicholls et al., 2011) with TEV protease recognition sequence (TevS-dark) 5'-actagtgagaatttgtacttccagggaccatgtaacgactcaagcg acccacttgttgtggcagcatcaattattggcattcttcacttaattctttggatcttggaccgtctttgactcgag-3' was synthesized with flanking SpeI and XhoI restriction sites. The EGFP fragment was amplified using primer pairs 5'-agaattcggatccatggtgagcaagggcgagg-3' and 5'-gactagtcttgtacagctcgtccatgcc-3'. The PCR product was digested with EcoRI / SpeI and the pUAST vector was digested with EcoRI/ XhoI. The TevS-dark, EGFP and pUAST were ligated to make pUAST-GFPdark construct. The

construct was injected using a standard P-element insertion method and multiple lines were generated. The line with the least background expression of GFP signal was used for this study.

The p10xUASTattB-3xHA-TEV construct: Full length TEV protease was first amplified with caccatgggagaaagcttgtttaag and ctcgagctagttcatgagttgagtcg primer pairs and then cloned into pENTER-D/TOPO vector. From TopoD vector the TEV protease then transferred into Gateway vector pTFHW (1123) to make N-terminal 3xHA tagged TEV protease. The 3xHA-TEV fragment was further amplified with agtcggtaccaacttaaaaaaaaaaatcaaaATGtacccatacgatgttcctgac and agtctctagactaGTTCATGAGTTGAGTCGCTTCCTTAACtgg and cloned into pJFRC81 vector (Addgene) to make p10XUASTattB-3xHA-TEV. The construct was then inserted at attP40 site in the 2nd chromosome.

pUASTattB-FKBPDD-Luciferase construct: The destabilized luciferase construct was made as described (Sellmyer et al., 2009) with following modifications. A fly optimized FKBP12L106P mutant variant was synthesized with TEV protease recognition sequence ENLYFQG at the c-terminal end. The fire fly luciferase gene was cloned in frame downstream of the TEV-protease recognition sequence.

pUASTattBJJJ2construct. The yeast JJJ2 was cloned into TopoD donor vector (Invitrogen). Using LR-clonase (invtorgen) JJJ2 was transferred to pUAST-HAattB vector (kindly provided by Dr. Konard Basler). The pUAST constructs were inserted in the attp40 site in the 2nd chromosome or attp2 site in the 3rd chromosome.

2.2 *Drosophila* strains

The following *Drosophila* strains are used in this study: Elav-Gal4 (stock no.458), ElavRU-Gal4 (stock no.43642), 201Y-Gal4 (stock no.4400), 17d-Gal4 (stock no.51631), MzVum-Gal4 (stock

no.29031), tubP-Gal80ts (stock no.7016), Cha-Gal80 (stock no.60321), Orb2RNAi (stock no.27050), UAS-GFP (stock no.1522), MB-Gal80 (stock no.64306), R11D09-Gal4 (stock no.48456). The stocks were obtained from the *Drosophila* stock center in Bloomington, Indiana.

2.3 RU486 feeding to induce TEV-protease expression

To induce expression of TEV protease using the GeneSwitchRU-Gal4 system we essentially followed the protocol described in McGuire et al. (McGuire et al., 2004) with some modifications. Briefly a 20mM stock solution of RU486 (Mifepristone, Sigma M8046) was prepared in 70% ethanol. The stock solution was diluted 1:20 in 2% sucrose (for courtship suppression memory) or distilled water (for olfactory appetitive conditioning) to a final concentration of 1mM. 70% ethanol diluted 1:20 in 2% sucrose solution (for courtship suppression memory) or distilled water (for olfactory appetitive conditioning) was used as vehicle control in feeding experiments. For olfactory appetitive conditioning, prior to training and testing flies were starved as groups in polystyrene vials (25 x 95 mm) containing Kimwipe soaked with 2.5mL 1mM RU486 for 18~22 hours. For male courtship suppression assay, flies were kept individually in polystyrene vials (25 x 95 mm) and before or after training exposed to a Kimwipe soaked with/without 2.5mL 1mM RU486 in 2% sucrose solution. In courtship conditioning to feed during training, 20mM stock solution of RU486 was diluted 1:40 in standard corn meal to a final concentration of 0.5mM. The flies were trained in 16 x 100 mm culture tubes (VWR) bottom of which were filled with the food. During no drug feeding period between training and testing, flies were kept in polystyrene vials containing standard corn meal. Where indicted, particularly in memory recovery experiments after training flies were kept in standard fly food for ≥ 2 hours before transferring to RU486 to ensure acquisition/encoding of memory.

2.4 Western Blot and Immunoprecipitation

For western blot analysis, fly heads were homogenized (2–4 µl of buffer/head) in a PBS buffer containing 150 mM NaCl, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5% glycerol, 0.1% Triton X-100, 1% NP40, and protease inhibitors (Roche), and approximately 3-5 head equivalents of extract were used. For the immunoprecipitation to detect Orb2 oligomer, 1.5–2 mg of total protein was incubated with 1 µg of the purified anti-Orb2 antibody 2233 (guinea pig) for 2 hr at 4°C and protein-A beads (Repligen) for an additional 2 hr. The IP then was blot with anti-Orb2 antibody 273 (rabbit).

Unlike monomeric Orb2, oligomeric/aggregated Orb2 is less abundant and the flies were fed 10uM tyramine to ensure detection of the aggregates. To quantify Orb2 oligomer/aggregates flies were fed 1mM RU486 + 10uM Tyramine in 2% sucrose solution or vehicle control+ 10uM Tyramine for 24h.

2.5 Yeast screening

For the yeast prion assay the nucleotide sequence of the n-terminal 160 amino acids of Orb2A were yeast optimized and fused in frame to Sup35 C-domain to create the chimeric construct Orb2Aprd-Sup35C. Using Gateway cloning strategy the chimeric construct was cloned into pAG414SUP35-ccdB-SUP35C (LEU, CEN plasmid, Sup35 promoter, SUP35C domain) Gateway vector (Invitrogen). The Orb2A-Sup35C (LEU selectable marker) construct was introduced into W303aΔsup35 strain [MATa; leu2-3, 112; his3-11,-15; trp1-1; ura3-1; ade1-4; can1-100; SUP35::HygB; [*psi*-];[*PIN*+] via plasmid shuffling. The yeast were grown in YPD media and plated on either YPD-agar or SC-agar lacking adenine and the [*PSI*+] colonies were selected 2–3 days after plating. Selected colonies were grown in YP-glycerol plates to avoid petites. To

determine frequency of prion-like conversion individual red or white colonies were grown in complete media and from a log phase culture 10X fold dilutions were plated in complete media and – Adenine plates. To determine heritability of prion or non-prion strains the colonies were streaked for multiple times. As we have reported previously (Hervas et al., 2016) the Orb2Aprd-SupC converts to the prion-like state in much higher frequency than Sup35. Therefore in all cases we observed some growth in –Adenine plates, especially in higher cell count. For the overexpression screen cells grown in 2% Raffinose to mid log phase were transferred to 2% Galactose or 2% glucose containing media to OD^{600} ~0.4 and grown overnight before plating into appropriate media.

Generation of yeast Hsp-deletions in Sup35 deleted background

The *S. cerevisiae* W303a cells lacking the Sup35 gene was kindly provided by Dr. Susan Lindquist (MIT). In this strain the essential Sup35 function was provided by the C-terminal fragment of the Sup35 gene (Sup35C) from a URA-based plasmid. To knockout individual non-essential Hsps in this background we have used the yeast deletion library. In this collection each yeast open reading frame is replaced with a KanMAx module, which allows for selection of the deletion strain in geneticin plates. Briefly we isolated genomic DNA from the Hsp::KanMAX deletion strains and PCR-amplified the cassette with a ~100bp extension in both side for homologues recombination. The Δsup35:Sup35P-SupC strains were transformed with the purified PCR fragments and the recombinants were selected in the geneticin plates. The deletion was verified by PCR using gene-specific and internal KanMAX primer pair followed by sequencing of the PCR product. Individual deletion strains were then transformed with Sup35P-Orb2Aprd-Sup35C plasmid with Leu-marker and via plasmid shuffling Sup35P-Sup35C was removed, resulting in Orb2Aprd-Sup35C being the only source of Sup35 protein. For overexpression, the Hsps were obtained from Yeast ORF

collection in BG1805 vector or HIP FLEXGene ORF collection in BY011 vector. In both vectors the Hsps are cloned under the Galactose inducible yeast Gal1 or Gal10 promoter respectively.

2.6 *In vitro* translation assay with JJJ2

The *in vitro* translation assay was performed as described by Khan et al (Khan et al., 2015). To perform *in vitro* seeding 5ng of Orb2A320 mRNAs or wild type or mutant JJJ2 mRNA were translated in WT or Δ*orb2* embryo extract for an hour at 26ºC and then the reactions were incubated at 4ºC for 24h. To test the effect of newly formed oligomer in translation, the Tequila translation reporters were pre-incubated for 30 mins with the oligomer and followed by translation in Δ*orb2* embryo extract for 30 mins.

The translation assay was carried out at 26ºC in 25 µL reaction volume, consisting of 50ng translation reporter, 40% (v/v) embryo extract, 16 mM Hepes-KOH, pH 7.4, 100 µM amino acid mixture (Promega), 250 ng/µl *S. cerevisiae* tRNA (Roche Applied Science), 50 mM potassium acetate, 2.5 mM magnesium acetate, 100 µM spermidine (Sigma), 20 mM creatine phosphate (Roche Applied Science), 80 ng/µl creatine kinase (Roche Applied Science), 800 µM ATP, and 100 µM GTP (Sigma). In all reactions 20U of RNase inhibitor (Invitrogen) was added prior to the addition of the translation reporter. Firefly and renilla luciferase activity was measured in 96-well plate reader (Perkin-Elmer 1420 Multilabel Counter) using the dual-glo luciferase assay system (Promega).

2.7 Single fly head luciferase assay

The flies were collected in a 1.5 ml Eppendorf tubes and snap-frozen in liquid nitrogen. The heads were separated from body by vortexing for 5-10 seconds and individual heads were transferred to the wells of 96-well flat-bottom micro-titter plate (Corning, NY, USA). The heads were then

crushed using pipette tips in 50 µl of PBS buffer containing 0.1% NP-40 (Sigma) and 0.1% Triton-X 100 (Sigma). 50 µL of luciferase substrate (Promega) was added in each well, incubated for 10 minutes at room temperature and luciferase activity was measured using a luminometer.

2.8 Male courtship suppression assay

The male courtship conditioning assay was modified from that described previously (McBride et al., 1999). Each male virgin was isolated right after eclosion in standard food vials. When they mature to 4~5 days old, each virgin male was paired with a freshly mated female for one to three sessions of 2 h each, with a 30 min rest period in between. During training sessions flies were kept in 16 x 100 mm culture tubes (VWR) provided with standard corn syrup fly food. Memory performance was tested with a fresh-mated female at the indicated time point in a 1 cm diameter wheel. A courtship Index (CI) was measured as the fraction of time the tested male spent chasing the female in a 10 min interval using an automated ImageJ based program. The Memory Index or courtship suppression index (Fig 6) was calculated as: $\frac{\overline{CI}_{Naive} - \overline{CI}_{Trained}}{\overline{CI}_{Naive}} \times 100$, where CI_{Naive} and $CI_{Trained}$ are the mean courtship indices for independent samples of naive and trained males, respectively.

2.9 Olfactory-Appetitive Conditioning

Flies were food deprived for 18 to 22 hour before conditioning in plastic vials containing kimwipes paper saturated with water. The wall of the training tube was covered with a Whatman filter paper saturated with 1M sucrose or indicated concentrations of sugar and a second tube was prepared similarly except that the filter paper was soaked in just water. Starved flies were introduced into the elevator of a T maze and tested in groups of 50-70 flies. Flies were transferred to the tube

containing sugar and exposed to an odor for 2 min. After 30 s of air stream, the flies were relocated in the elevator and shifted to the tube without sugar in the presence of the second odor for 2 min. For the 24hr test, flies were given standard cornmeal food for 3hr after training. They were then transferred to plastic vials containing a kimwipe soaked with water and starved for 17hr before testing. For the 48hr memory test, flies were given standard cornmeal food for 18–24hr after training and then were starved for 24–30 hr prior to testing. During the memory test, flies were introduced into the elevator and transported to a point where they have to choose between two air streams, one carrying the reward associated odor and the other with the non-associated odor. Animals were given 2 min to choose between the two odors. Different group of flies were trained in a reciprocal experiment in where the -reward/+reward odor combination were reversed (3-Octanol or 4-Methylcyclohexanol). The performance index (PI) is calculated as the number of flies in the reward odor minus the number of flies in the non-reward odor, divided by the total number of flies in the experiment. A single PI value is the average score of the first and the reciprocal experiment.

2.10 Image acquisition and quantification

To image the GFPdark signal, immediately after testing the flies were anesthetized and the brain was dissected into PBS. Images were acquired using a Zeiss LSM 510 Meta system in regular PMT imaging mode. The 488 laser was used to excite GFP through a HFT405/488/543 dichroic. Emission was reflected by a NFT 545 dichroic and through a BP 505-530 nm emission filter. A 20X, 0.8 NA plan apochromatic objective was used. Z-step size was 2.0 um. the pinhole was set to 53 um.

All analysis was performed in ImageJ. After background subtraction, each frame was spatially binned 2x2 and smoothed. To measure intensity in the α/α', β/β', and γ lobes, regions of interest

(ROIs) were generated by hand over a representative, uniform region in the respective lobe, and average intensity was recorded. Z profiles over the respective region were analyzed to ensure the maximum intensity z-slice was used. GFP intensity in the γ lobe was normalized to the GFP intensity in the β/β' lobes to reduce the noise of variation in GFP-dark expression level. To eliminate potential bias in manual selection of ROIs, all data analysis was performed blindly on randomly named data sets: the analysis was done without the knowledge of 1) the genotype of the fly, 2) its memory score, and 3) what group the brain originated from, trained or untrained.

High resolution imaging to detect GFP-dark fibers (Fig S10D) was performed on a Zeiss LSM 780 confocal microscope equipped with an LD C-Apochromat 40x 1.1NA objective, a 40 um pinhole (1 Airy Unit), and a pixel size of 145 nm. Stacks were collected at a spacing of 700 nm and line averaging of 2. Excitation utilized a 488 nm laser with an MBS 488 dichroic. Detection was accomplished with the GaAsP spectral detector in integration mode and a gain of 788 in the wavelength range from 499 to 543 nm.

2.11 Number of trials (n) and Statistical analysis

All statistical analysis was performed using Graphpad Prism 5. All of the data met the assumption of homogeneity of variance, therefore unpaired two-tailed t-test or one-way analysis of variance (ANOVA) was performed, Tukey post-hoc test between pairs of samples. ANOVA tests for significance were performed at a probability value of 0.05 and more stringent values are listed in each figure where applicable. For all experiments, each *n* is considered a biological replicate; separate trials used independent samples of genetically identical flies. In olfactory training experiments a single *n* is approximately 100-140 flies. Based on previous and ongoing experimental effect sizes, 8-10 of double trials were generally judged to be adequate for memory experiments, unless effect sizes were strikingly large or variable. For courtship conditioning the n

indicates number of individual male flies used in that group. In all long-term memory experiments, experimental manipulations for which a negative result was plausible or expected were always trained alongside a positive control.

For the correlation test between courtship suppression index and GFP intensity, flies with positive courtship suppression index were plotted and fit with linear regression, and the p-value and R^2 shown were also based on the population of positive courtship suppression index. Please see the supplemental table for the entire data set. In the mock trained and 1x trained group, flies either did not form any memory or had low memory. Therefore the number of flies with positive courtship suppression is ~50% of the total number of flies tested. In mock trained or 1X trained group the flies with high courtship suppression index represents random distribution of courtship activity,

2.12 Monte Carlo correlation analysis

In order to test the statistical reliability of the correlation between memory index and GFP-dark ratio, we performed a Monte Carlo analysis (Bevington, 2003). Given that such analysis is strongly dependent on the shape of each variables statistical distribution, we chose a methodology which makes no assumptions for this shape. The method randomly shuffles the intensity ratio measurements and then assigns them to the unshuffled memory indices. This random shuffling was performed 100,000 times and each shuffle was fit with linear regression to create a probability distribution of slopes. The analyses were performed using custom written Java code available at http://research.stowers.org/imagejplugins. As expected, the distribution is centered at 0 and the probability was reported as the fraction of simulated slopes which were greater than or equal to the experimental value.

Chapter 3. Orb2 activity in the mushroom body gamma neurons is necessary for persistence of long-term memory

3.1 *Drosophila* as a model organism to study learning and memory

In the 1900s Thomas Morgan introduce the vinegar fly (also referred to as fruit fly) *Drosophila melanogaster* as a powerful genetic model. Through a forward genetic screen, Morgan and his students isolated a series of heritable mutants with abnormal morphology of the eyes and wings (MORGAN, 1910). In the 1960s Seymour Benzer introduced the same idea to study the interaction between genes and specific behavior (Benzer, 1967). There are two far reaching consequences of the studies initiated by Benzer and his colleagues. First, their work led to the discovery that the flies can perform many types of behavior, from simple phototaxis to complex Pavlovian associative memory. The inventions of these behavior paradigms laid the foundations for future behavioral research in *Drosophila*. Second, Benzer and his colleagues isolated many "first" genes critical for different behaviors, such as *period* for circadian rhythms, *dunce* for learning and memory, *fruitless* for courtship, etc., each of which opened up molecular dissection of specific behavior (Benzer, 1971). The isolation of the first batch of learning and memory mutants converge on to the cAMP-CREB pathway (Davis, 2005) shared with other systems such as *Aplysia* (Schacher et al., 1988) and rodents (Bourtchuladze et al., 1994) (see also Chapter 1). These early research set *Drosophila* as a pioneering model to study interplay between genes, circuit and memory.

3.2 Memory paradigms for study of long-term memory in *Drosophila*

Following the pioneering work of Benzer and colleague, in a relatively short time a variety of behavioral paradigms were developed, such as olfactory conditioning (aversive,

appetitive and proboscis extension reflex), visual learning, heat box, courtship suppression and predator avoidance. Of them, olfactory conditioning and courtship suppression are the most widely used memory paradigm to investigate especially long-term memory.

3.2.1 Associative olfactory conditioning

Olfaction is critical for the survival of insects since it is crucial for foraging, mating and predator avoidance. Therefore, odor often serves as a strong associative cue. In the laboratory condition, there are two associative olfactory conditioning paradigms that are widely used because they produce robust memory that lasts for days.

The aversive associative memory paradigm is the earliest memory paradigm for *Drosophila*, developed in the Benzer's laboratory. In this paradigm a group of flies are alternately exposed to two neutral odorants (conditioned stimuli) and one of them is coupled with electric shock (unconditioned stimuli). Memory was tested as flies' avoidance to shock-associated odor (Quinn et al., 1974). Subsequently Tim Tully made this paradigm more useable by modifying it into a binary T-maze choice conditioning assay (Fig 3.1A) (Tully and Quinn, 1985). In this training, 1 min presentation of an odor (CS+) with 12 pulses of electric shocks (1s each with 5s interval), followed by 30s of rest without CS or US, and then another 1 min presentation of a second odor without electric shock (CS-). The flies are then transported to the T-maze where they can make a choice between tubes containing CS+ and CS- for 2 min. A memory index (MI) is calculated by subtracting the number of flies in the CS+ tube from the number of flies in the CS- tube, divided by the total number of flies. A second group of flies is trained similarly except the shock-associated odor is reversed to control for intrinsic odor biases. The final MI is calculated as the average of the two reciprocal conditioning. The beauty of this experimental system is one can manipulate the strength of US to control the stability of memory. One session of training usually

generates memory that lasts up to 3h post training. While 6-10 sessions of trainings produce memory that persists for days (Tully et al., 1994).

The odor avoidance paradigm was very successful in identifying some of the key molecular pathways involved in memory. However, the strength of memory was variable and memory decayed quite rapidly (Tempel et al., 1983). The appetitive associative memory paradigm trains a group of hungry flies to associate neutral odors with sugar as a reward (Fig 3.1B). The neutral odorants (3-Octanol or 4-Methylcyclohexanol) serve as conditioned stimuli (CS) and rewarding sugar as unconditioned stimuli (US). One major difference from the aversive training is that starvation of the trainee flies is prerequisite for the memory to form as well as for the flies to retrieve the memory during test. Wild type flies starved between 16-20h before conditioning can form appetitive association. In this paradigm, a group of flies are exposed to the first odor (CS+) in a tube lined with sucrose (US) solution soaked filter paper for 2 min, followed by a 30s break, then exposed to a second odor (CS-) in a separate tube lined with water soaked filter paper for 2min. This simple transient pairing of CS-US can surprisingly generates memory lasting for days (Krashes and Waddell, 2008; Tempel et al., 1983). The key components are the starvation, which motivates the flies to learn and form memory, and the quality of sugars used as US. For example, sweet nutritious sugars such as sucrose and fructose produce long-term memory, while sweet but non-nutritious sugars such as xylose produces short-term but no memory beyond 24h (Burke and Waddell, 2011).

The simple behavioral paradigms and powerful genetics led to the discovery of genes and circuits that contributed significantly to our understanding of memory. On the gene level, the first set of genes isolated with *Drosophila* aversive olfactory conditioning were involved in the cAMP pathways: dunce, the cAMP phosphodiesterase (Dudai et al., 1976) and rutabaga, an adenylyl

cyclase (Livingstone et al., 1984a). Later more genes were identified in other pathways or affecting memory at later time points (Drain et al., 1991; Yin et al., 1994b). On the anatomical level, it established the role of insect mushroom body as the center for learning and memory, and more recently mapping of mushroom body input and output neurons lead to the better understanding of how brain integrates CS-US and differentiates the valiancy of different stimuli (Keene and Waddell, 2007). New research also starts to emerge specially to understand the interaction between memory and other physiological processes such as aging (Tonoki and Davis, 2015), sleep (Dissel et al., 2015) and immune system (Babin et al., 2014).

3.2.2 Male courtship suppression conditioning

Another widely used training paradigm in *Drosophila* is the male courtship suppression conditioning. Developed in 1979 (Siegel and Hall, 1979), this paradigm was based on the observation that male courtship is modified by prior sexual experience. Mature naïve males court mature females vigorously and display a sequential movement towards the female: orienting, tapping, singing, licking and copulation (Sokolowski, 2001), whereas males rejected by unreceptive fertilized females for an extended time show less interest towards all females. The training starts with isolating naïve male into individual tube to avoid any social interaction among flies that can potentially modify courtship. After 4-5 days, when the male fly is sexually matured, it is paired with a fresh mated unreceptive female for 1-2h. During the training periods, the male receives repeated rejection from the mated female. Like olfactory conditioning, the strength of training determines the persistency of memory. One training session produces memory persisting for couple of hours (Siegel and Hall, 1979). Three sessions of training with 30min interval can generate memory lasting for days (McBride et al., 1999). Courtship conditioning differs from olfactory conditioning in two ways: first, courtship conditioning is a modification of innate

behavior, which may explain its longer training time than olfactory conditioning, because to inhibit or reverse an instinctive behavior requires more training, while the olfactory conditioning tries to connect unrelated information without reversing the hard-wired program; second, the courtship conditioning is a single fly assay and rules out the group effect during decision making, it also allows to score variations of flies' ability to produce memory. Because courtship behavior of male is a complex sequential behavior incorporating the innate drive and feedback from female, recruiting olfaction, vision, and touch sensory modalities (Yamamoto and Koganezawa, 2013), the underlying genes and neuronal circuit are not yet fully elucidated. Remarkably, the gens and circuits that control male courtship suppression memory overlap largely with olfactory conditioning, suggesting independent memories could converge into same genes and circuits (McBride et al., 1999). Male courtship conditioning also provides unique opportunity to study how different sensory systems work in concert to represent a memory.

3.3 *Drosophila* brain anatomy underlying memory

The *Drosophila* brain contains ~100,000 neurons with the general feature of scattered cell bodies in the outer brain surface and clustered neuropil enriched in the inner region (Chiang et al., 2011). Like the mammalian brain, neuropils are segregated for different physiological functions. These organized structure dictates a variety of complex behaviors including learning and memory, courtship, foraging and sensorimotor coordination. For each brain hemisphere, 29 distinct segments could be identified (Armstrong et al., 1995; Chiang et al., 2011), the most distinguishable ones among them are antennal lobe, medulla, mushroom body, subesophageal ganglion, ellipsoid and fanshaped body, controlling olfactory, vision, memory, motor control and sleep respectively. Mushroom body neurons are extensively studied in the context of learning and memory and its role in the persistence and recall of memory is well documented (Davis, 2011). Other brain regions

such as central complex that composed of ellipsoid body, fanshaped body and protocerebral bridge have also been reported to modulate memory but only a subset.

Mushroom body

Mushroom bodies are a pair of most distinct structures in the insect brains. It is an evolutionary conserved structure in Arthropod (Strausfeld et al., 1998) and has been postulated to be functionally equivalent to cerebral cortex, thalamus and hippocampus of the vertebrate brain. However, the gross structural differences between vertebrate brain and insect brain suggest that the mushroom bodies and certain vertebrate brain regions are unlikely derived from the same origins or developed under the control of homologous genes (Strausfeld et al., 1998). Despite their structural differences, both hippocampus and *Drosophila* mushroom body mediated memories are dependent on an overlapping set of molecules (Kandel and Abel, 1995), suggesting the molecular engram likely to be more conserved than the circuit engram.

The mushroom body was first described in 1850 by the French biologist Felix Dujardin. Its morphology was better characterized with Golgi staining by F.C. Kenyon in 1896, who named the structure “Mushroom Bodies” based on the long stalk crowned with a cap of cell bodies (Kenyon Cells) (Kenyon, 1896). For his contribution mushroom body neurons are also referred to as “Kenyon Cells”. The function of mushroom body was connected to intelligent behavior based on the observations that species with larger mushroom body tend to have more complex behavior. Early trials in ants, cockroach, butterflies and honeybees suggest mushroom bodies are involved in spatial memory (Mizunami et al., 1993; Sivinski, 1989; Vowles, 1964) and olfactory conditioning (Menzel et al., 1974). In the early 1980s, influenced by the powerful mutagenesis and memory paradigm developed by Benzer’s lab, efforts were made to identify mutants that show impaired mushroom body morphology and study the memory of these mutants (Heisenberg et al.,

1985). This not only confirms the functional connection between mushroom body and memory, it also provides methodology to systematically investigate in detail how memory is processed in this structure or even substructure, how memory related molecules work in the mushroom body neurons, and in broad how different memories are encoded and modified.

Cell bodies of Kenyon cells, are clustered at dorsal brain. They send short dendritic fibers that converged to make up the calyx. Kenyon cells also send axons in bundle (pedunculus) towards the ventral part where the axon fibers bifurcate with one branch growing vertically (α lobe) and the other growing medially (β lobe) (Heisenberg, 1998). The general organization of mushroom body neuropil is now defined as α/β lobes, α'/β' lobes and ϒ lobe (Crittenden et al., 1998). α and α' lobes project vertically, while β, β' and ϒ lobes project horizontally towards the midline. All the lobes are extended from the heel structure (Fig 3.2).

Although the lesion study and mushroom body mutants confirm that mushroom body is a structure important for memory, memory has different phases (encoding, storage and retrieval) and the permanent damage do not allow for temporal analysis. This necessitates methodology that allows transient interference of the neuronal function. Through combination of different Gal4 lines and expression of transgenes that reversibly blocks synaptic transmission at elevated temperature (shibire-ts)(Kitamoto, 2001), the function of different mushroom body lobes can be dissected out during different phases (encoding, storage and retrieval) of memory. Using this methods, it was revealed that mushroom body is dispensable during encoding and storage of memory but their output to downstream neurons are critical for retrieval of memory in the short term (Dubnau et al., 2001; McGuire et al., 2001). With more detailed characterization of distinct mushroom body lobe Gal4 lines, it is suggested that in olfactory conditioning mushroom body output from α/β, α'/β' and ϒ lobes contribute to the retrieval of short-term memory up to 3h; memory that lasts to 24h is

solely dependent on output from α/β while the other lobes become dispensable (Cervantes-Sandoval et al., 2013).

Another approach is to use genetically encoded molecular sensors for functional optical imaging, and compare the activity of neurons before and after conditioning. One of the most popular tools for functional optical imaging is the calcium sensor G-CaMP, which is engineered to increase its fluorescence upon influx of calcium (Nakai et al., 2001). G-CaMP allows visualization of neuronal activity at different time point after training and in multiple areas of the brain. With this technique, it was identified that a memory trace formed in the axon branch of α'/β' lobes 5min after olfactory conditioning and persist for 60min (Wang et al., 2008), establishing an early memory trace in the α'/β' lobes. A long term memory trace that becomes detectable 9h post olfactory conditioning is located to the α/β lobes in conditions that only generate long-term memory. This trace persists to 24h and decays afterwards (Yu et al., 2006). Remarkably, this long-term memory trace display strongly in the α branch, consistent with the phenotype of the mushroom body structure mutant alpha-lobes-absent (ala) that specifically shows defective long-term memory (Pascual and Preat, 2001). The mushroom body ϒ lobe is reported to store a memory trace beyond 24h (Akalal et al., 2010).

Because of simple cues and robust memory, most of the circuit mapping is done using the olfactory conditioning as described above. To summarize, the α/β lobes are essential for long-term memory (Blum et al., 2009), the α'/β' lobes are required for intermediate-term memory (Krashes et al., 2007), while the role of γ lobe neurons is still controversial, with some evidence suggest in short-term memory (Blum et al., 2009; Zars et al., 2000a) but others suggest in long-term memory (Akalal et al., 2010). This is perhaps due to different time scales examined between the

experiments and it could be that γ neurons are utilized to encode short-term memory and again required to maintain/express memories that beyond 24h.

The memory circuit in other types of conditioning shares a lot with olfactory conditioning especially in the mushroom body intrinsic neurons but with some deviations. For example, the ϒ lobe neurons are required for both encoding and retrieval of visual memory while other lobes are dispensable (Vogt et al., 2014); the γ-lobe neurons are also important for short-term taste memories (Kirkhart and Scott, 2015). In male courtship suppression memory paradigm, ablation of mushroom body impairs with both short- and long-term memory (McBride et al., 1999). And later it was identified that synaptic signaling in the α/β and α'/β' lobes are needed for courtship conditioning (Montague and Baker, 2016; Redt-Clouet et al., 2012). Interestingly, unlike olfactory conditioning, multiple evidence suggest ϒ-lobe neurons are involved specifically in storage and expression of long-term courtship suppression memory (Fitzsimons et al., 2013; Fitzsimons and Scott, 2011; Keleman et al., 2007; Krüttner et al., 2012), however, these studies were not directly targeting the neurons, they were instead based on requirement of different molecules in the γ-lobe neurons, such as histone deacetylase (Fitzsimons et al., 2013; Fitzsimons and Scott, 2011), CaM kinase (Joiner and Griffith, 1999) and CPEB(Orb2) (Keleman et al., 2007; Krüttner et al., 2012). These results suggest that mushroom body γ-lobe neurons contain a cascade of molecular activity required for courtship suppression memory.

3.4 *Drosophila* CPEB and memory

The powerful genetics of *Drosophila*, combined with established memory paradigm and long-searched memory circuit, makes it a Swiss army knife for study genes and memory. However, elucidation of genes that function specifically in long-term memory through forward genetic screens is still labor-intensive (Walkinshaw et al., 2015), therefore, a lot of the genes that

regulate long-term memory are identified or confirmed by targeted genetic manipulation. Same with *Drosophila* CPEB. It has long been speculated that the CPEB protein family might be specifically involved in protein-synthesis dependent long-term memory based on their characterized roles in translation regulation during oogenesis and wide expression in the nervous system (Wu et al., 1998). However as there are four CPEB genes in mammals, knock-out of one gene does not lead to obvious memory deficit (Berger-Sweeney et al., 2006; Chao et al., 2013; Tsai et al., 2013), possibly due to compensating effect from other CPEBs. *Drosophila* has two CPEB genes: Orb (mammalian orthologue CPEB1 and *Xenopus* CPEB) and Orb2 (mammalian orthologue CPEB2-4). Both Orb and Orb2 have reported roles in regulation of mRNA translation during oogenesis (Lantz et al., 1992) and spermatogenesis (Xu et al., 2012) respectively, and they also express in the central nervous system (Keleman et al., 2007; Pai et al., 2013). Interestingly, unlike in mouse, knock-down or knock-out of either Orb or Orb2 in *Drosophila* leads to long-term memory deficit (Keleman et al., 2007; Pai et al., 2013). These results suggest each *Drosophila* CPEB has critical role in memory that cannot be compensated once down-regulated. Therefore, *Drosophila* serves as a good model to study CPEB-dependent long-term memory.

3.5 Objectives and rationale

Previous studies suggest oligomerization of Orb2 is necessary for the maintenance of long-term memory (Khan et al., 2015; Majumdar et al., 2012) and the function of it is restricted to mushroom body γ neurons (Krüttner et al., 2012). However, whether Orb2 is involved in neurons outside mushroom body and how its biochemical property functions to coordinate the circuit activity is not understood. The first step to tackle this problem is to map the neurons in which Orb2 activity is required for long-term memory. To this end I have used male courtship suppression

memory paradigm and UAS-Gal4 system to restrict the Orb2RNAi expression in subpopulation of neurons. These tools will allow me to map the Orb2-dependent neurons within and outside mushroom body.

3.6 Orb2 activity is required at 48h post training

To determine the time course of Orb2 activity in the maintenance of memory, I utilized the male courtship suppression memory paradigm because multiple evidence suggested Orb2 activity was required for this memory (Keleman et al., 2007). In this paradigm a single male fly is isolated until 4-5 days after eclosion. During training, each male fly is paired with an unreceptive female for 2h. Three training sessions allow the male fly to learn to suppress its courtship after repeated rejection by the unreceptive female and this memory can last for 9 days (Fig 3.3A) (McBride et al., 1999; Siegel and Hall, 1979). To suppress Orb2 expression, I first used a broad Gal4 line MzVum Gal4. Originally characterized in the larval CNS, the MzVum Gal4 labels ~80% of the neurons in the adult brain including mushroom body, fan-shaped body, central complex and medial bundle neurons (Fig 3.3B). Knocking down Orb2 expression in these neurons by driving expression of double stranded RNA against Orb2 (Orb2-RNAi) did not affect memory at 5min and 24h post training. However, when measured 48h post training the memory was significantly impaired (Fig 3.3C). In the control groups such as MzVum Gal4 crossed to wild type (MzVumGal4/+) or UAS-Orb2 RNAi crossed to wild type (Orb2RNAi/+), stable memory was observed at all time points (Fig 3.3C).

3.7 Mapping of Orb2 dependent memory neurons with Gal4 and RNAi intrinsic to the mushroom body

Next I sought to determine whether mushroom body neurons, particularly whether specific lobes of mushroom body are involved in Orb2-dependent memory. To this end I used 201Y Gal4 line, which shows expression in mushroom body α/β and γ lobes to drive Orb2 RNAi (Fig 3.4A). Expression of Orb2A RNAi in the mushroom body neurons using 201Y-Gal4 resulted in a memory deficit when measured 2 days after training (Fig 3.4B). Similar to 201Y, expression of Orb2RNAi under another γ lobe specific Gal4 line R11D09 (Fig 3.4A), resulted in memory impairment (Fig 3.4B). In contrast, expression of Orb2RNAi using the mushroom body Gal4 line 17D, which only expresses in α/β lobe (Fig 3.4A), had no effect on memory (Fig 3.4B). These results suggest Orb2 is required in the mushroom body γ-lobe neurons for memory beyond 24h.

To further confirm that it is the γ lobe neurons, I took advantages of another genetic tool, the Gal80 system. Gal80 is a transcription repressor of Gal4 from budding yeast and has been introduced into the flies to refine the expression of Gal4 (Suster et al., 2004). For example, ChaGal80 (expression of Gal80 in cholinergic neurons) when combined with 201Y Gal4 suppresses the Gal4 expression in the mushroom body ϒ lobe while leaves the α/β unaffected (Fig 3.4A). Knocking down Orb2 in these neurons does not affect memory (Fig 3.4C). Similarly, MBGal80 which suppresses 201Y Gal4 expression specifically in the mushroom body (Fig 3.4A) also has no effect on memory when crossed with Orb2 RNAi (Fig 3.4C). Taken together, these results suggest Orb2 activity in the mushroom body ϒ lobe but not α/β lobe is required for memory to persist at 48h.

3.8 Mapping of Orb2 dependent memory neurons with Gal4 and RNAi outside the mushroom body

If Orb2 activity is restricted exclusively to the mushroom body, subtracting the mushroom body expression from the MzVum neurons (Fig 3.5A) and knocking down Orb2 in neurons outside

the mushroom body neurons should not have any effect on memory. Surprisingly, expressing Orb2 RNAi in MzVumGal4-MBGal80 flies, I still observed a memory defect at 48h (Fig 3.5B). This result suggests that a subpopulation of MZvumGal4 positive neurons outside the mushroom body are also important for memory. To further identify these MzVum positive neurons outside mushroom body, I combined MzVumGal4 with ChaGal80, which prevents Orb2 RNAi expression in mushroom body ϒ lobe, as well as cholinergic neurons outside mushroom body (Fig 3.5A). Under these conditions there was no memory defect (Fig 3.5B), suggesting that the Orb2 dependent neurons outside the mushroom body is likely to be cholinergic. In addition to mushroom body neurons a recent study that used a temperature sensitive RICIN to block protein synthesis suggested a pair of neurons located at the dorsal-anterior-lateral (DAL) protocerebrum (Fig 3.5A) are required for protein synthesis dependent long-term memory (Chen et al., 2012). These neurons however, do not show Orb2 dependent memory (Fig 3.5B). Which neurons mediate the Orb2 dependent memory outside mushroom body remains elusive. Nevertheless, my observation poises an interesting direction to identify a parallel memory trace outside mushroom body but within the MzVum and cholinergic neuron population (Fig 3.5C).

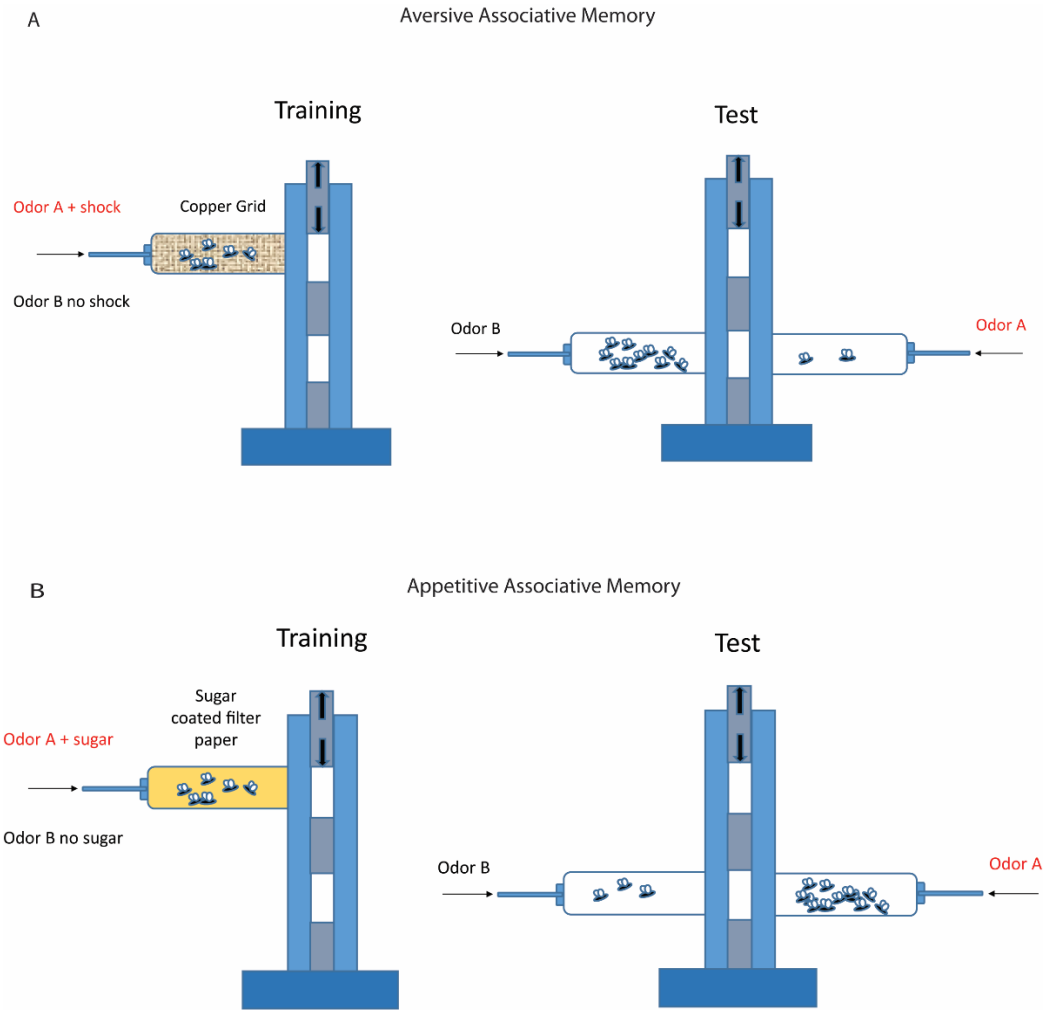


Figure 3.1 Pavlov olfactory conditioning in *Drosophila.*

The Pavlov classic olfactory conditioning can pair odor (conditioned stimuli) with either aversive or appetitive unconditioned stimuli. **A)** In aversive associative conditioning, a group of flies (~100) are exposed to odor A paired with pulses of electric shock for 1 minute, and then exposed to odor B without electric shock after 30s gap. During test, the flies are sent to a T-maze where they can choose to go towards odor A or B. If the flies form aversive memory, they should avoid odor A. **B)** In appetitive associate conditioning, flies are prestarved and then during training exposed to odor A in a tube coated with sugar. After 2 minutes, they are given a 30s rest and then exposed to odor B in a fresh tube without any sugar. The test is done the same way as aversive conditioning, except that the flies need to be starved before test. If appetitive memory is formed, more flies should go towards odor A.

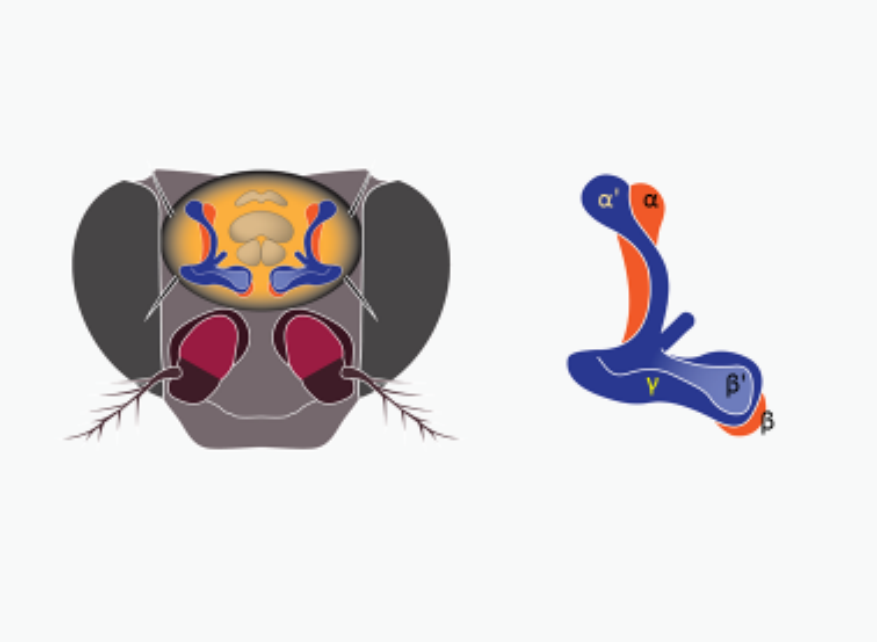


Figure 3.2 *Drosophila* mushroom body.

Dorsal view of a fly head highlighting the position of the mushroom body. A closer view of the mushroom body shows the Kenyon cells in the calyx send axons in a bundle towards the ventral part where the axon projections bifurcate into vertically projected α and α' lobes and horizontally projected β, β' and ϒ lobes towards the midline. The α and β lobes (orange) are originated from the same bundle, same with the α' and β' lobes (light purple). While the ϒ lobe (dark purple) extends only horizontally.

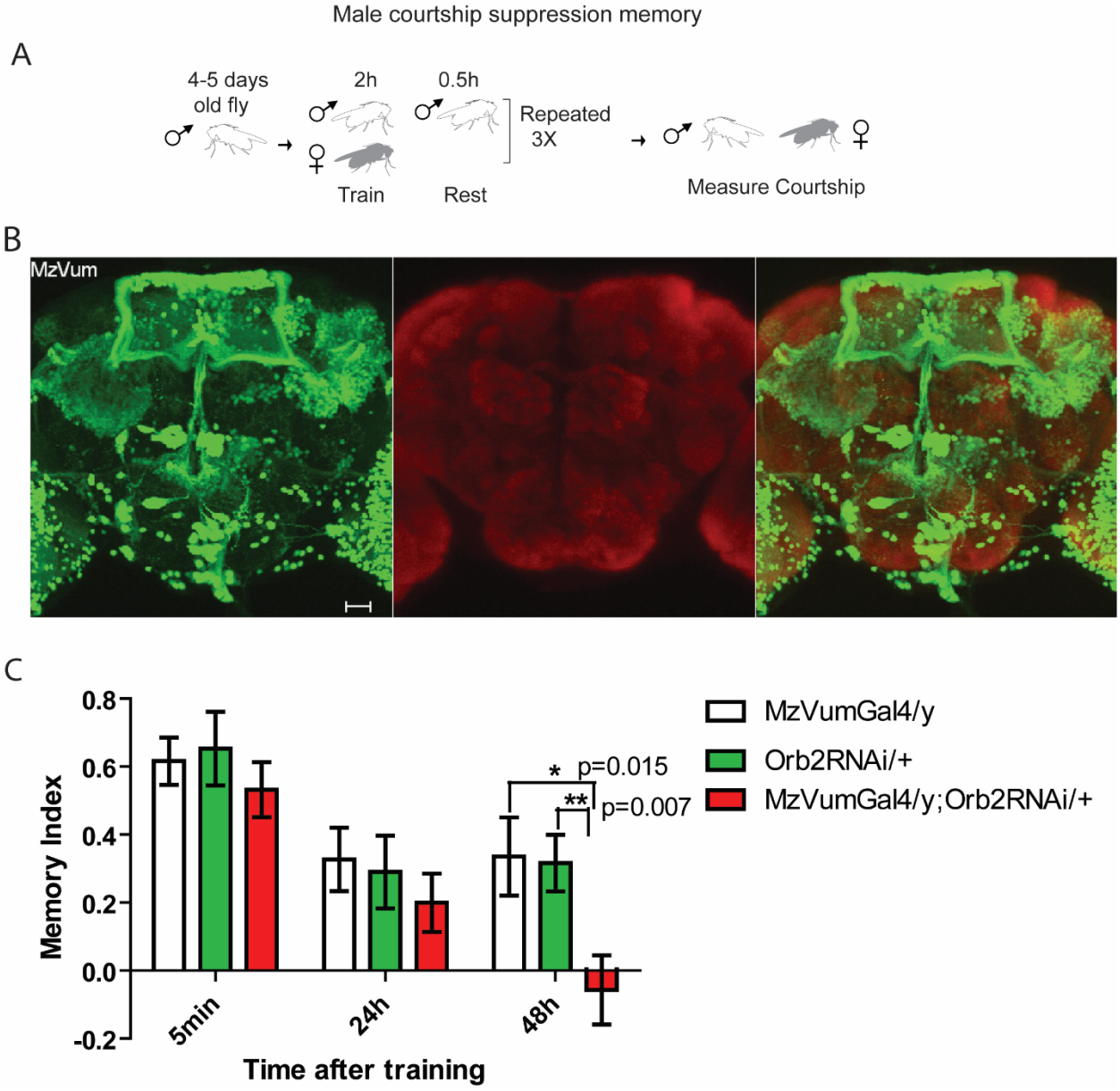


Figure 3.3 Orb2 is required in *Drosophila* male courtship suppression memory.

A) Schematic of the male courtship suppression memory paradigm. Virgin males were isolated for 4-5 days prior to training and then exposed to a freshly mated female for 2h. Then the male was left alone for 30min before the next training session with another mated female. The test was done at indicated time point after training by measuring the male courtship activity towards another unreceptive female.

B) Expression pattern of MzVum neurons. Fly genotype is MzVumGal4 > UAS-CD8GFP. Green channel is the GFP positive neurons labeled by MzVumGal4. Red channel is a presynaptic active zone protein Bruchpilot (nc82). Scale bar: 20um.

C) Knock-down of Orb2 in MzVum neurons interferes with male courtship suppression memory at 48h post-training. Memory is measured at 5min, 24h and 48h after training. *$p < 0.05$, **$p < 0.01$.

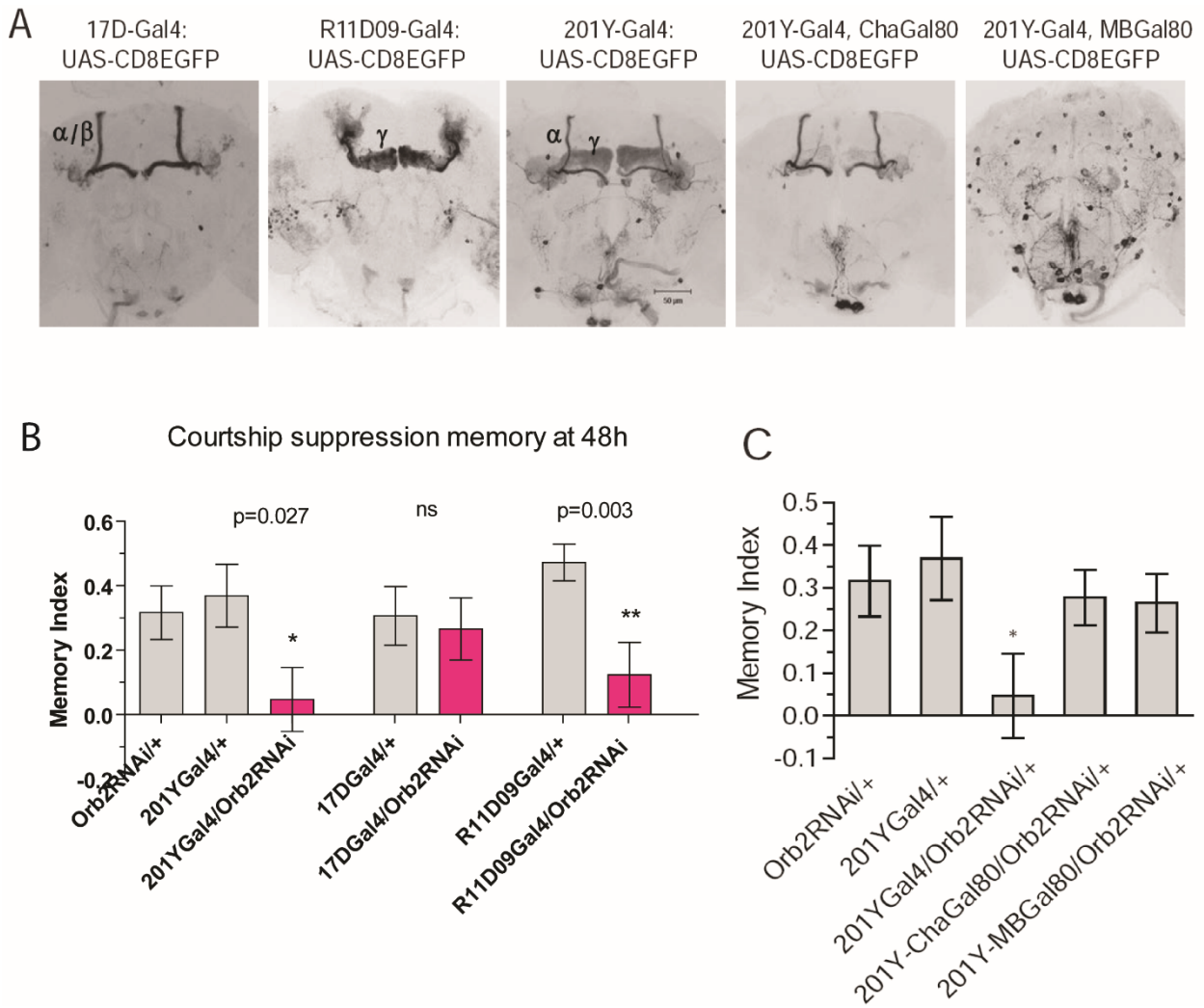


Figure 3.4 Mapping of Orb2 dependent memory neurons in the mushroom body.

A) Expression pattern of various Gal4 driver lines used. 201Y-Gal4 and R11D09-Gal4 drive expression in. 17D-Gal4 expresses only in the α/β lobes. Cha-Gla80 and MB-Gal80 suppress expression in the γ-lobe neurons. Scale bar 50um.

B) Suppression of Orb2 expression in γ-lobe neurons with 201Y-Gal4 and R11D09-Gal4 suppress memory at 48h. Suppression of Orb2 expression in the α/β lobes with 17D-Gal4 does not lead to memory defect.

C) Suppression of Orb2RNAi expression in the γ-lobe neurons with Cha-Gal80 and MB-Gal80 rescues long-term courtship suppression memory.

*$p < 0.05$, **$p < 0.01$.

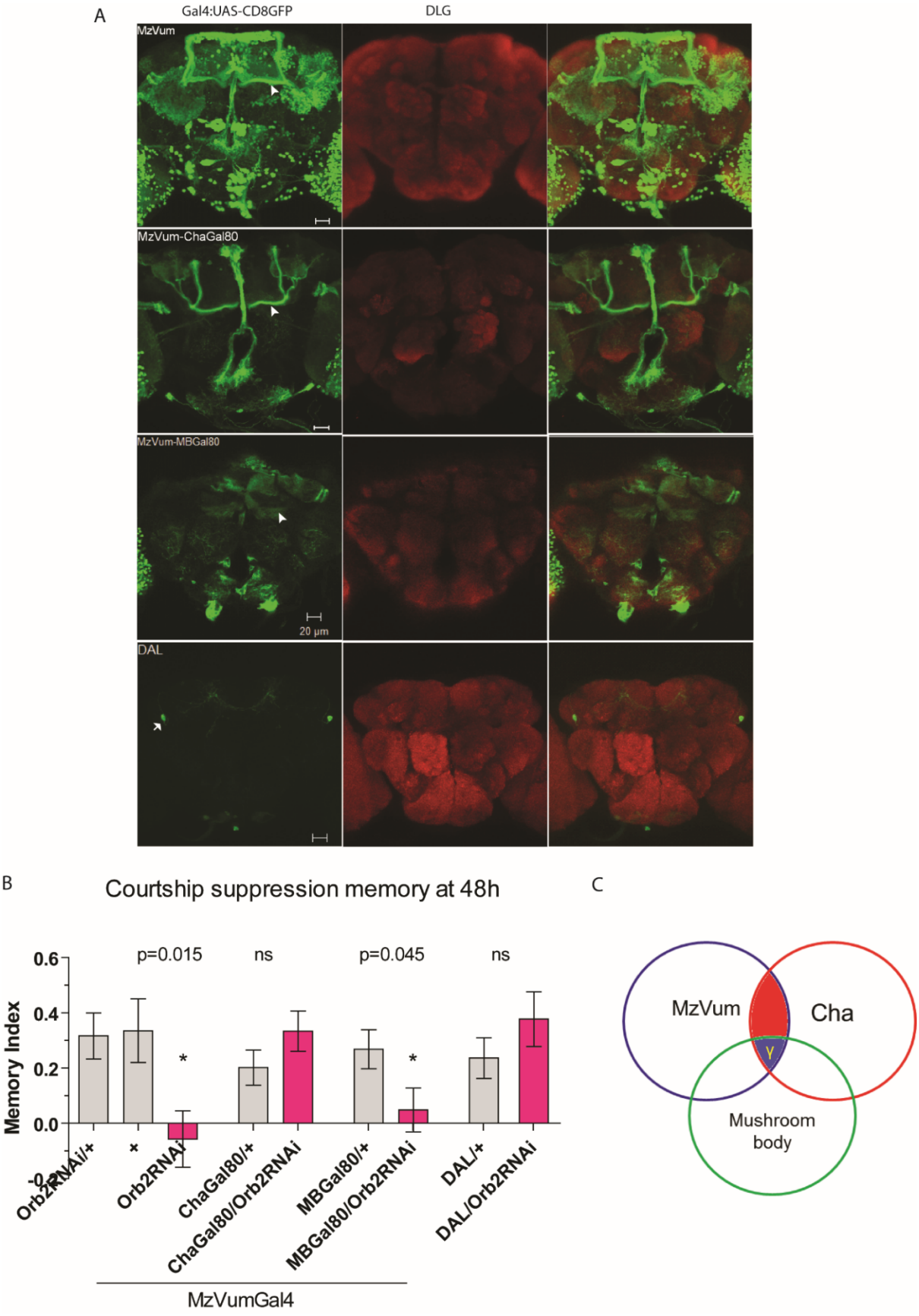

A
Gal4:UAS-CD8GFP
DLG
MzVum
MzVum-ChaGal80
MzVum-MBGal80
20 µm
DAL
B
Courtship suppression memory at 48h
Memory Index
0.6
0.4
0.2
0.0
-0.2
p=0.015
ns
p=0.045
ns
*
*
Orb2RNAi/+
+
Orb2RNAi
ChaGal80/+
ChaGal80/Orb2RNAi
MBGal80/+
MBGal80/Orb2RNAi
DAL/+
DAL/Orb2RNAi
MzVumGal4
C
MzVum
Cha
Y
Mushroom
body

Figure 3.5 Mapping of Orb2 dependent memory neurons outside the mushroom body.

D) Expression pattern of MzVum-Gal4 in combined with Cha-Gla80 and MB-Gal80, and also the two dorsal-anterior-lateral (DAL) neurons. Green channel shows membrane-bound GFP expressed by Gal4. Red channel is a post-synaptic protein disc large (DLG-4F3). Scale bar 50um.

E) Suppression of Orb2 expression outside the mushroom body also leads to memory defect at 48h. But Orb2 activity is not required in the dorsal-anterior-lateral (DAL) neurons, rather it is required in cholinergic neurons.

F) Summary of the Orb2-dependetn neuron mapping indicates both γ neurons within mushroom body and cholinergic ∩ MzVum neurons outside mushroom body are critical for long-term memory in an Orb2-dependent manner.

*$p < 0.05$, **$p < 0.01$.

Chapter 4. Orb2 is required for encoding and retrieval of long-term memory

4.1 Objectives and rationale

Previous studies suggested Orb2 is required for the persistence of memory. This conclusion was based on the observation that both Orb2 deletion mutant and Orb2-oligomer defective mutant fail to maintain memory beyond a day (Keleman et al., 2007; Majumdar et al., 2012). However, while these experiments established a role of Orb2 in long-lasting memory, they failed to define exactly which aspect (s) of long-term memory was particularly dependent on Orb2. Memory is composed of different phases -- encoding, storage and retrieval -- and disruption of any of these steps would result in behavior manifested memory impairment. To determine the requirement of Orb2 in various phases of memory requires a method to transiently inactivate the Orb2 protein in the nervous system. Gene deletion/mutation or RNAi were not useful for this purpose since 1) they create a chronic depletion of the protein, 2) inducible RNAi has a slow kinetics, and 3) they rely on normal decay of the existing proteins, whereas aggregates of Orb2 are stable (White-Grindley et al., 2014b) requiring inactivation of the existing protein, not just elimination of new Orb2. Keeping these issues in mind I first developed genetic tools that allow rapid and reversible inactivation of Orb2 protein in the neurons and then using these reagents to ask when Orb2 activity is required- encoding, storage, or retrieval.

4.2 A system for rapid and reversible inactivation of *Drosophila* Orb2 protein.

Various techniques have been described for the purpose of inducible inactivation of specific proteins *in vivo*. There are three main classes: inhibition of protein activity by small peptide inhibitor, induction of protein degradation, and protease cleavage of protein. Inducible

expression of small peptide inhibitor is genetically simple and does not require modification of the target protein. However, specificity is always the underlying concern. For example, the use of PKMξ inhibitor ZIP not only targets PKMξ but also PKC-ι and PKC-ζ (Lee et al., 2013). Furthermore, such inhibition although can be rapidly induced, is not necessarily reversible and may dependent on protein conformation. This raises the necessity to have multiple evidence when applying peptide inhibitor. Induction of protein degradation, such as deGradFP (Caussinus et al., 2012), proteolysis-targeting chimaeras (PROTACs) (Sakamoto et al., 2001) and auxin-inducible degron (Osada et al.) system (Nishimura et al., 2009), is recently becoming popular across different systems. These methods all involve tagging target proteins with specific tags that can be brought to proteasome for degradation. Proteins can be specifically, rapidly and sometimes reversibly degraded. However, these systems all require making N- or C-terminus fusion target proteins, which might interfere with the function or conformation of a protein. Orb2 has N-terminal prion-like domain and C-terminal RNA binding domain, both of which are sensitive to tags, making these strategies not optimal. Site-specific protein cleavage by matching protease stands out for its specificity and small protease recognition site that can be inserted at ideal residues (Parks et al., 1994; Volkmann et al., 2012). The tobacco etch virus (TEV) protease is the most widely used because of its specificity (Dougherty et al., 1989), low toxicity and high catalytic activity over a broad pH and temperature ranges (Kapust et al., 2001). In flies, the TEV protease cleavage system has been successfully applied (Harder et al., 2008; Pauli et al., 2008), showing specificity and no observed toxicity.

Therefore, I decided to develop a TEV protease based Orb2 protein inactivation system. The Orb2 protein has two isoforms, Orb2A and Orb2B: they share the same RNA binding domain and majority of the prion-like domain (Fig 4.1A). Because modification on the RNA binding

domain is more likely to interfere with its RNA binding activity, I decided to insert the recognition sequence of TEV protease, ENLYFQG (TevS) before the RNA binding domain, including the prion-like domain. This experiment serves two purposes: one, to find a site that can be efficiently cleaved; two, to survey the protease accessibility across the prion-like domain. I therefore modified the Orb2A protein because it is a critical determinant for aggregation of endogenous Orb2 (Majumdar et al., 2012). The *Drosophila* S2 cell system allowed me to quickly test the cleavage efficiency and accessibility of Orb2A prion-like domain without making transgenetic flies. I expressed various Orb2A-TevS constructs in S2 cells and incubated the cell lysate with/without TEV protease (Fig 4.1B). TEV protease does not have any effect on un-modified Orb2. Insertion of TevS at amino acid (aa) 23 and 197 likely reduces the stability of the Orb2A proteins since they were expressed at low level; insertion of TevS at aa137/160/216 shows 100% cleavage; aa144/197 are partially cleaved; only a small fraction of aa88 is cleaved and there is no reduction of the full length protein upon TEV cleavage. Interestingly, aa23 shows two distinct immunoreactive species: a higher molecular weight one resistant to TEV cleavage and a lower molecular weight one that migrates at predicted size and is fully cleaved by TEV protease (Fig 4.1B). These observations are consistent with the idea that the prion-like domain shows conformational plasticity.

I chose Orb2A aa216 for further *in vivo* analysis for the following reasons: one, it does not interfere with the protein stability; two, TEV protease fully cleaves the protein; three, the site sits in between prion-like domain and RNA-binding domain, less likely to interfere with prion-like properties and RNA binding; four, cleavage results in two detectable fragments, n-terminal prion-like domain and c-terminal RNA binding domain, allowing me to ask the consequences of separating these two parts. I therefore introduced a genomic fragment containing TevS (aa 370 for Orb2B and aa 216 for Orb2A) modified Orb2 (Orb2TevS) in an *orb2* null background (Fig 4.1A).

This modified Orb2 (Orb2TevS) is functionally equivalent to wild type Orb2 (Khan et al., 2015), rescues all orb2 deficiencies, and TEV protease cleaves and renders both monomeric and oligomeric Orb2TevS translationally inactive (Hervás et al., 2016; Khan et al., 2015). To determine whether this system could reversibly deplete Orb2 protein in an inducible manner *in vivo*, I used a RU486 inducible Gal4-UAS system to express HA-tagged TEV-protease (Fig 4.2A). In this system, the Gal4 transcription factor is fused to a progesterone ligand binding domain. The Gal4 is inactive in absence of the ligand. Since *Drosophila* lacks progesterone or progesterone-like hormone, the system can be induced by feeding flies with RU486 (mifepristone, a competitive progesterone receptor antagonist) (McGuire et al., 2004; Roman et al., 2001). I crossed UAS-HA-TEV flies with ElaveGeneswitchGAl4, an inducible Gal4 that specifically express in the neurons (Fig 4.2A). Feeding of 1mM RU486 within 4-6 hrs induced HA-tagged TEV protease and resulted in a gradual decrease of Orb2 protein (Fig 4.2B, B1). After 24 hours there was a ~50% reduction in both forms of Orb2 (0.50±0.06 in monomer and 0.45±0.10 in oligomer) (Fig 4.2B- B2 and B3). Upon removal of the RU486 the TEV protease level dropped significantly (0.79±0.07,n=4) within 24 hours (Fig 4.2C, C_2) with a corresponding increase in monomeric Orb2 protein level almost to the wild type level (0.82±0.17) (Fig 4.2C, C_1). The relatively rapid disappearance of TEV protease upon RU486 withdrawal is likely due to the limited period of RU486 exposure and short half-life of TEV-protease. Taken together these results suggested the Orb2 - TEV system can be used to transiently decrease the Orb2 protein level from adult neurons.

4.3 Orb2 activity is required for consolidation and stable maintenance of long-term memory

Previous studies of mutant of Orb2 (Keleman et al., 2007; Majumdar et al., 2012) and my results of Orb2 RNAi (see Chapter 3) suggest Orb2 is required for long-term memory consolidation and maintenance. To determine whether transient depletion of Orb2 protein can

manifest with any of these behavioral consequence, I designed the following assays to ask if Orb2 is involved in memory consolidation or maintenance, respectively.

First, to assay memory consolidation, flies were exposed to 1mM RU486 or to vehicle only as a control and long-term memory was assessed in male courtship suppression paradigm (Fig 4.3). In this paradigm a male fly learns to suppress its courtship behavior for days after repeated rejection by an unreceptive female (Fig 4.3A)(McBride et al., 1999; Siegel and Hall, 1979). Compared to vehicle controls, flies fed with RU486 before, during and immediately after training had significantly less memory after a day (Fig 4.3B) consistent with previous studies (Kacsoh et al., 2015; Keleman et al., 2007; Majumdar et al., 2012). Feeding of RU486 to genetic controls had no effect on long-term memory (Fig 4.3E).

Second, to assay stable maintenance of long-term memory, flies were trained and allowed memory to consolidate. Then flies were given either RU486 or vehicle immediately after (Fig 4.3E), one day after, or 2 days after training, and memory was tested at the end of 24 hours of RU486 feeding (Fig 4.3C). Compared to the vehicle, feeding of RU486 at any time after training resulted in a significant reduction ($p<0.05$) in memory score after a day (Fig 4.3C&E). To distinguish between the possibilities that 1) the RU486 fed flies have never formed any memory or 2) they have formed memory but failed to maintain and/or retrieve it, a group of flies were tested 24h after training to ensure memory formation and then exposed to RU486 for 24h then tested a second time. Exposure to RU486 resulted in a significant reduction in already formed memories (Fig 4.3D).

The above observations suggest transient depletion of Orb2 proteins lead to deficit in memory consolidation and maintenance in male courtship suppression memory. To test whether such continued dependency on Orb2 manifests in different forms of memory (Kacsoh et al., 2015;

Majumdar et al., 2012), I used the associative appetitive memory paradigm (Krashes and Waddell, 2008; Tempel et al., 1983) in which hungry flies learn to associate a particular odor (conditioned stimulus, CS) with a rewarding food (unconditioned stimulus, US) following a 2-minute pairing and the preference for CS lasts for days (Fig 4.4A). Similar to the courtship suppression paradigm flies fed with RU486 before or immediately after training or one or 2 days post-training resulted in a significant loss in memory within the subsequent 24h (Fig 4.4B). Feeding of RU486 did not interfere with memory of flies expressing only TEV-protease or of flies with modified Orb2TevS but absence of TEV protease (Fig 4.4C). Taken together these results suggest Orb2 activity is required for memory consolidation and days after memory formation in at least two distinct memories.

4.4 Orb2 activity is required for encoding and retrieval of long-term memory

At a given test time point, if a memory cannot be retrieved, there are 3 possibilities: one, the memory is not encoded; two, the memory is encoded but failed to be stored; and three, the memory is encoded and stored properly, but the retrieval process is blocked. The absence of behavioral manifestation of memory upon depletion of Orb2 protein could be due to any of the possibilities. The transient Orb2 inactivation system allowed me to investigate these possibilities that cannot be achieved with mutant flies.

As figure 4.3A shows, flies fed with RU486 from -24h to +24h show memory defect at 24h time point. To distinguish whether this phenotype is due to a defect in encoding or retrieval, I further limit the feeding of RU486 between -24h to +2h. Based on the Orb2-TEV cleavage kinetics, the Orb2 level is less than 50% during entire training process and 6h post-training, a time frame critical for protein-synthesis dependent encoding of long-term memory. Then I withdraw the flies from RU486 and let them recover on standard food, allowing re-expression of Orb2 (Fig 4.5A).

Compared to control flies, RU486 treated flies show memory defect at 24h post-training, even when the Orb2 protein level is normal. The memory is still defective even when tested at 48h post-training (Fig 4.5A). These results suggest Orb2 is necessary during encoding of long-term memory. If a memory is not encoded, presence of Orb2 during memory retrieval is not sufficient to express a memory.

Next I asked if a memory is successfully encoded, is Orb2 required for memory to be retrieved. Although the results above suggested Orb2 is involved in maintenance of long-term memory after it is consolidated, it's not clear whether depletion of Orb2 protein leads to removal of the stored memory or the memory is still stored in the brain but cannot be retrieved. If the stored memory is disrupted, subsequent re-expression of Orb2 will not be sufficient to retrieve the same memory. While if the stored memory is intact but reduction in Orb2 protein level is not sufficient to retrieve a memory, then subsequent restoration of Orb2 protein may allow the memory to re-express. To distinguish between these two possibilities, flies were trained to allow memory consolidated to 24h, then exposed to RU486 for a day, and then removed from RU486 to restore the Orb2 protein; memory was tested after 12h and 24h (Fig 4.5B). Unlike the vehicle treated group that maintained their memories in all time points tested, for flies that formed memories 24h after training, their memory was reduced upon exposure to RU486 as expected; however, surprisingly, memory gradually recovered upon withdrawal of RU486 (Fig 4.5B). To verify that memory indeed changed in the same fly, in an independent set of experiment, memory score of each individual fly at three time points after training was plotted: before exposure to RU486, 24h after RU486 treatment, and 24h after withdrawal of RU486 (Fig 4.5C) and observed a similar loss and recovery of memory in individual flies. The memory recovery is not a consequence of multiple testing (which may reinforce memory), since testing only once at the end showed similar recovery

as multiple testing (Fig 4.5D). Similarly, recovery is not because memory has become independent of Orb2, since following memory recovery (Fig 4.5E, left) inactivating Orb2 again before testing resulted in memory impairment (Fig 4.5E, right). These observations suggest that Orb2 is required for retrieval of memory, and reduction of Orb2 after memory establishment results in transient amnesia.

My experiments did not address whether Orb2 is required for storage of memory. Because the TEV protease cleavage does not remove 100% of the Orb2 protein. The experiment to directly address the necessity of Orb2 for storage of a memory is to temporally but completely deplete Orb2 from the system between encoding and retrieval and then re-express. I have tried to design such a system by removing the Orb2 from genome then artificially re-express it. Unfortunately, the Flp-FRT system that is wildly used to flip-out the gene works at low efficiency in the Orb2 locus in the adult brain. However, the Kandel group has performed similar experiment with the mice prion-like CPEB3 and found that completely removal of CPEB3 gene locus leads to permanent memory loss, even when CPEB3 is re-expressed in the brain by virus injection (Fioriti et al., 2015). This results indicate the prion-like CPEB is required to store a memory. See Chapter 7 discussion also.

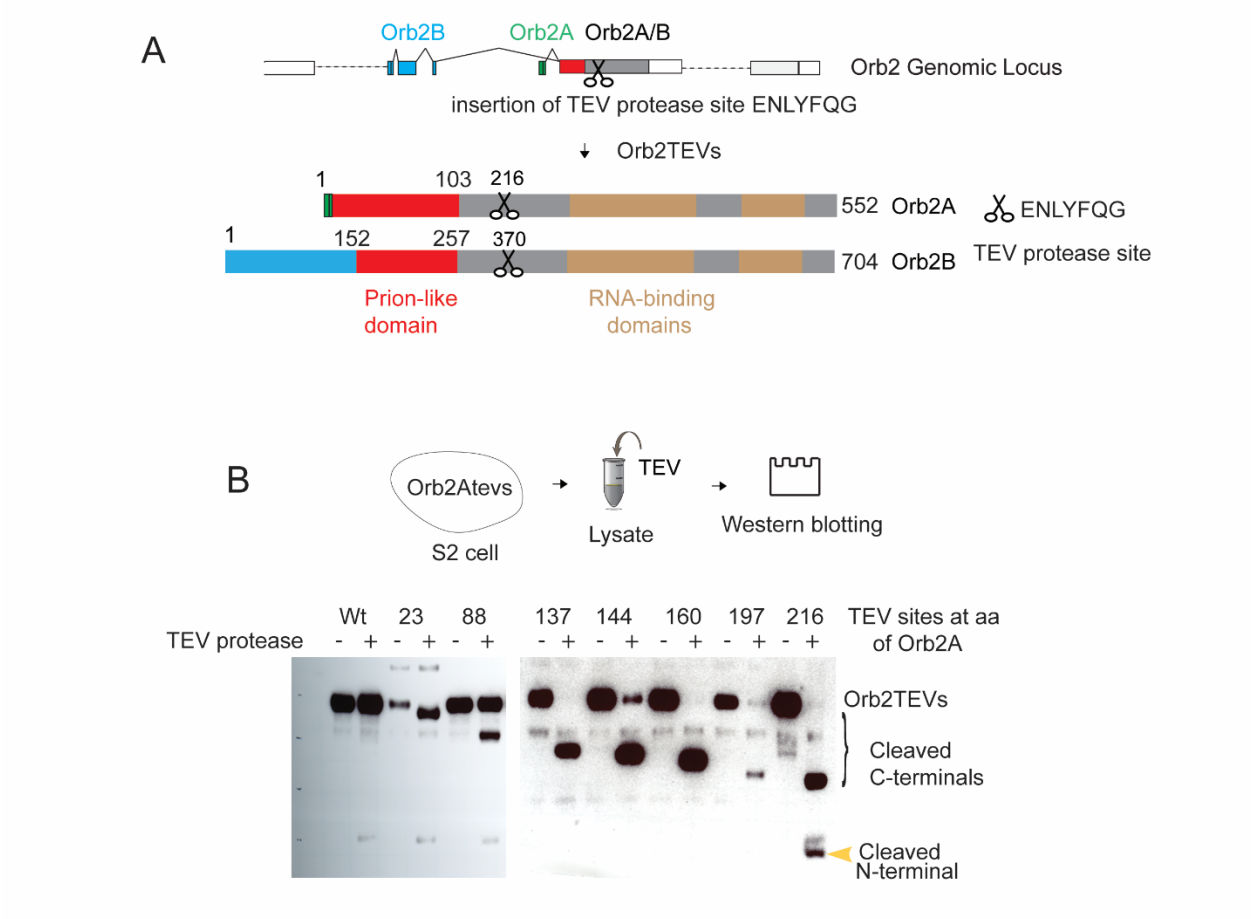


Figure 4.1 Screen of the Orb2 cleavage by TEV-protease.

A) Schematic of the Orb2 genomic locus and two isoforms Orb2A and Orb2B. Both Orb2 proteins are composed of prion-like domain and RNA binding domain. The position of the TEV-protease recognition site (TevS216) is indicated.

B) A S2 cell based screen of various TevS insertions in Orb2A protein that can be cleaved by TEV protease. The TevS was inserted in Orb2A protein at indicated positions. The modified Orb2A protein were expressed in S2 cell and total cell lysate was treated with 1ug of TEV protease overnight at 4 °C and analyzed in western. The bracket include cleaved C-terminal fragment of different sizes. The yellow arrow head points to N-terminal fragment of cleaved Orb2TevS(216). The faint band in wt, 23 and 88 is not an N-terminal fragment, they are immunoreactivity to *in vitro* added TEV protease.

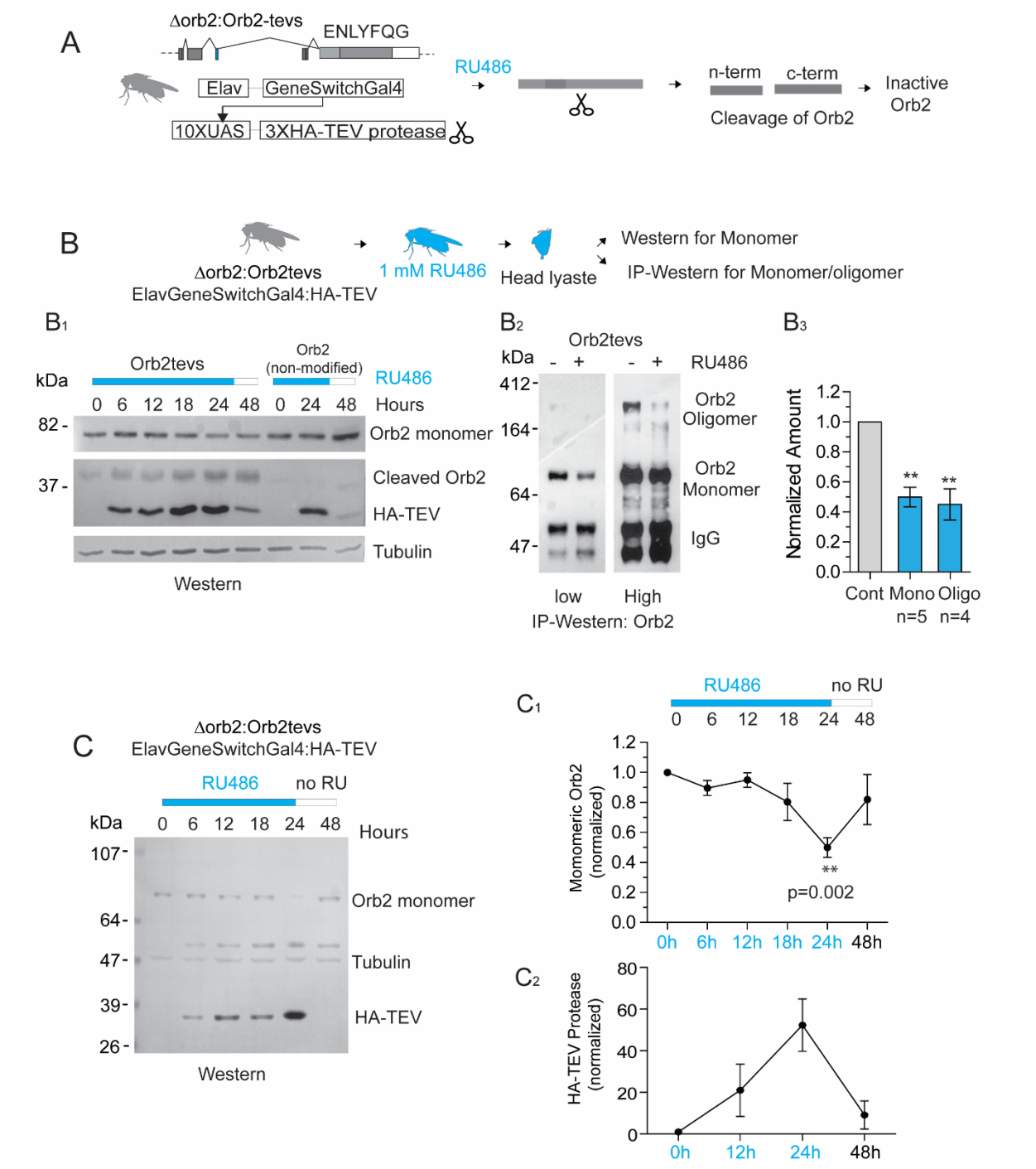


Figure 4.2 A system to acutely and reversibly inactivate *Drosophila* Orb2 protein.

A) Schematic of the experimental design. An Orb2 genomic fragment bearing a TEV protease recognition sequence (TevS) ENLYFQG was introduced in *orb2* null flies and expression of HA-tagged TEV protease was induced in neurons by RU486 to cleave and inactivate Orb2TevS protein.

B) Orb2TevS monomer (left panel) and oligomers (middle panel) are cleaved by TEV protease *in vivo*. (B1) Orb2TevS and non-modified Orb2 were fed with RU486 (blue bar) to induce TEV protease expression then retrieved from RU486 (white bar) to reduce TEV expression. Samples were collected at indicated time point. (B2) Orb2TevS were fed with RU486 for 24h and subjected to IP-western. Two different exposures of the same gel are shown to illustrate the reduction in both monomer (left) and oligomer (Wright and Dyson) level. (B3) Quantification of monomer and oligomer level after 24h of RU486 feeding normalized to vehicle control.

C) Recovery of monomeric Orb2 level following removal of RU486. Western blotting (left panel) and quantification (right panel) of total monomeric Orb2 protein (C1) and TEV protease (C2) following 1mM RU486 feeding (blue bars) and 24h after withdrawal of RU486 (white bar). The data are expressed as mean±sem and statistical significance was determined by using one way ANOVA (tukey multiple comparison) for more than two samples or unpaired t-test for two samples. The ** indicates $p \leq 0.01$ and *** indicates $p \leq 0.001$.

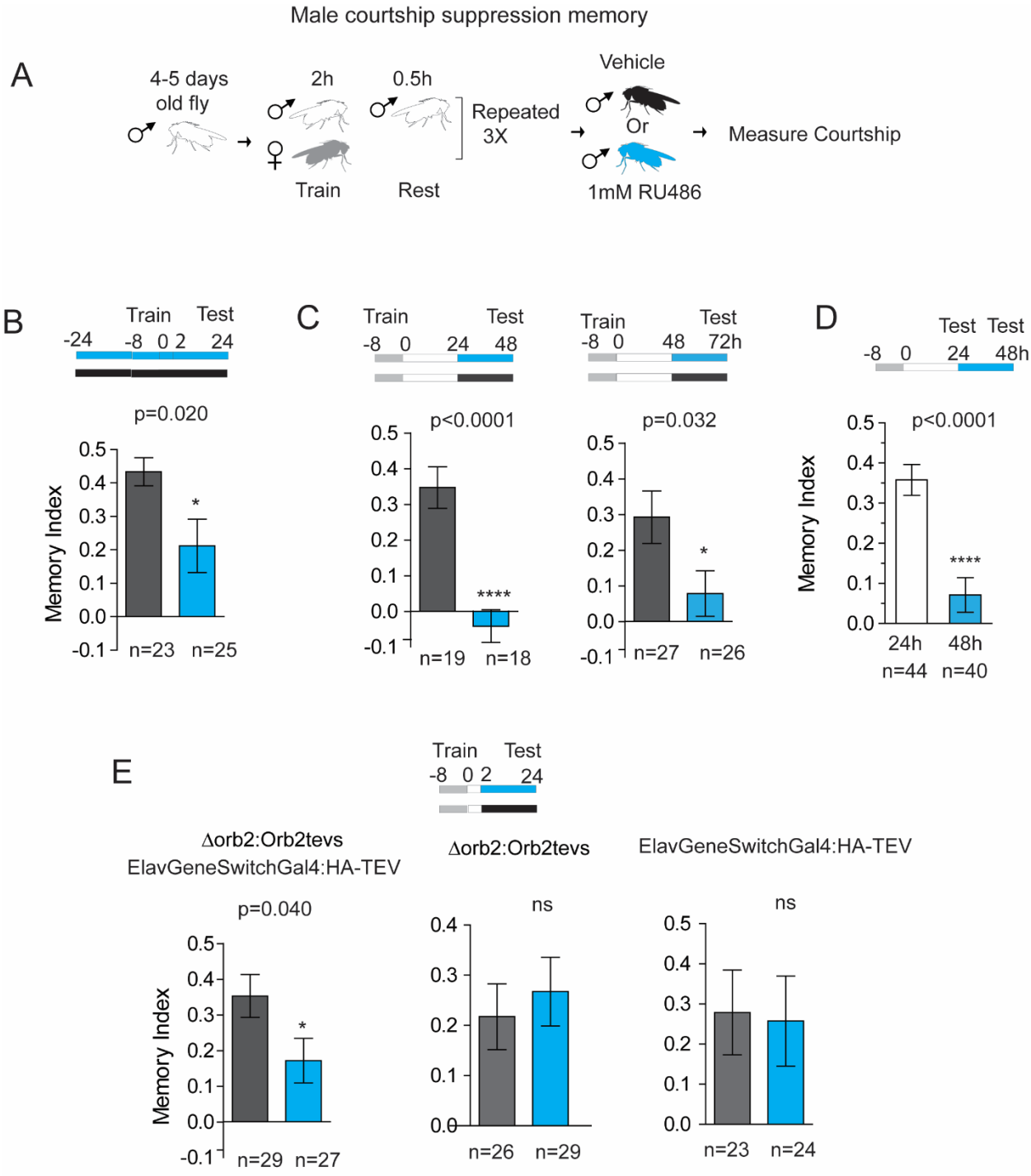


Figure 4.3 Orb2 activity is required for consolidation and maintenance of long-term memory in courtship suppression memory.

A) Schematic of the male courtship suppression memory paradigm. Virgin males were isolated for 4-5 days prior to training and then exposed to a freshly mated female. Flies were either fed with 1mM RU486 (blue) or vehicle before testing with another mated female.

B) Feeding of RU486 before, during and immediately after training reduces memory.

C) Feeding of RU486 any time after training reduces memory. Schematic of RU486/vehicle feeding at different time period post courtship suppression training (top) and the memory index after 24h of feeding (bottom).

D) RU486 feeding reduces existing memory. The same sets of flies were tested before (24h) and after feeding (48h).

E) RU486 or TEV-protease does not interfere with long-term memory. Flies with indicated genotype were fed with RU486 (blue) or vehicle (black) for 24h post-training. Only in flies expressing both modified Orb2 and TEV protease significant loss in memory was observed.

Memory index for each group was plotted as mean ± SEM and statistical significance was determined by unpaired t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

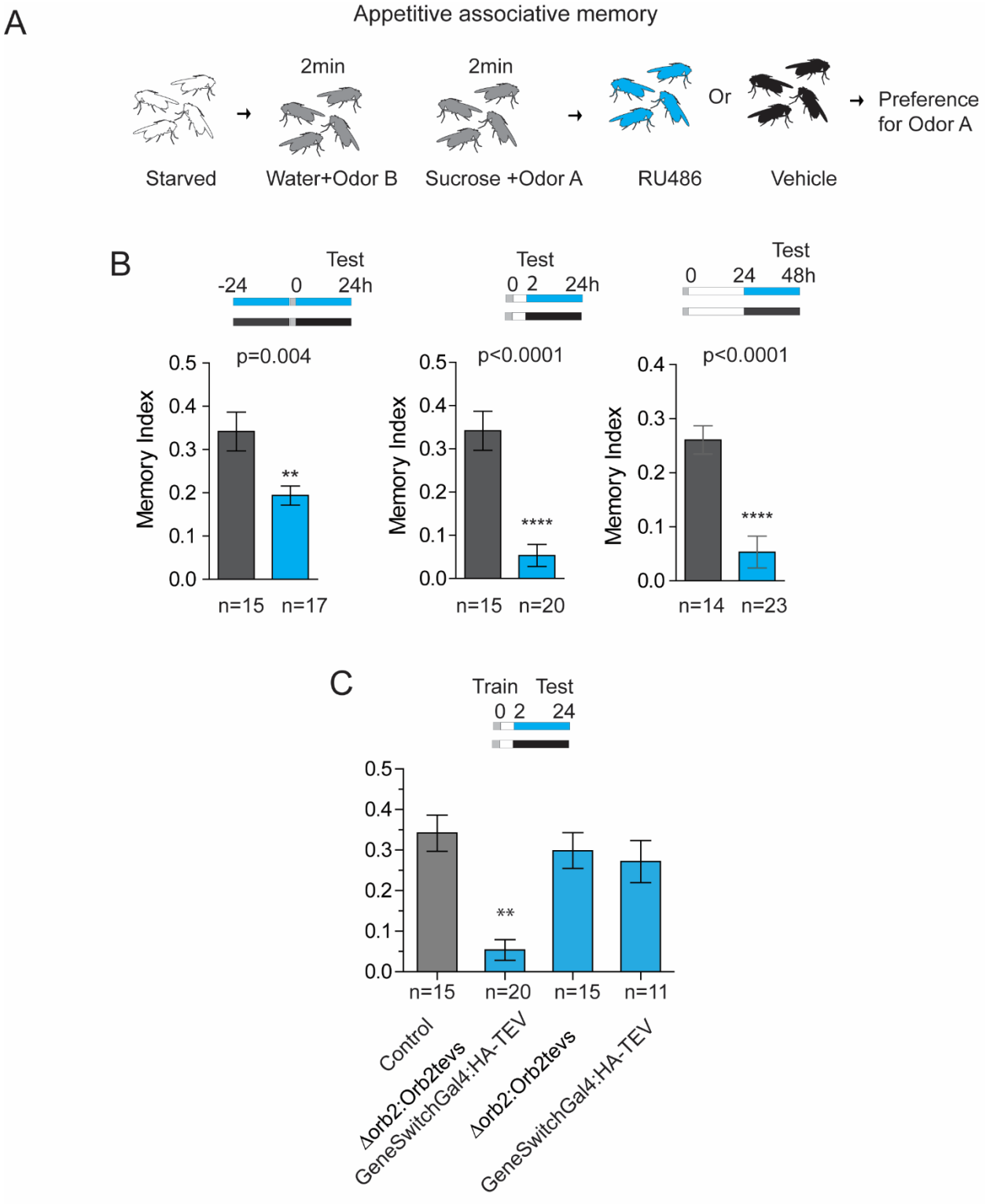
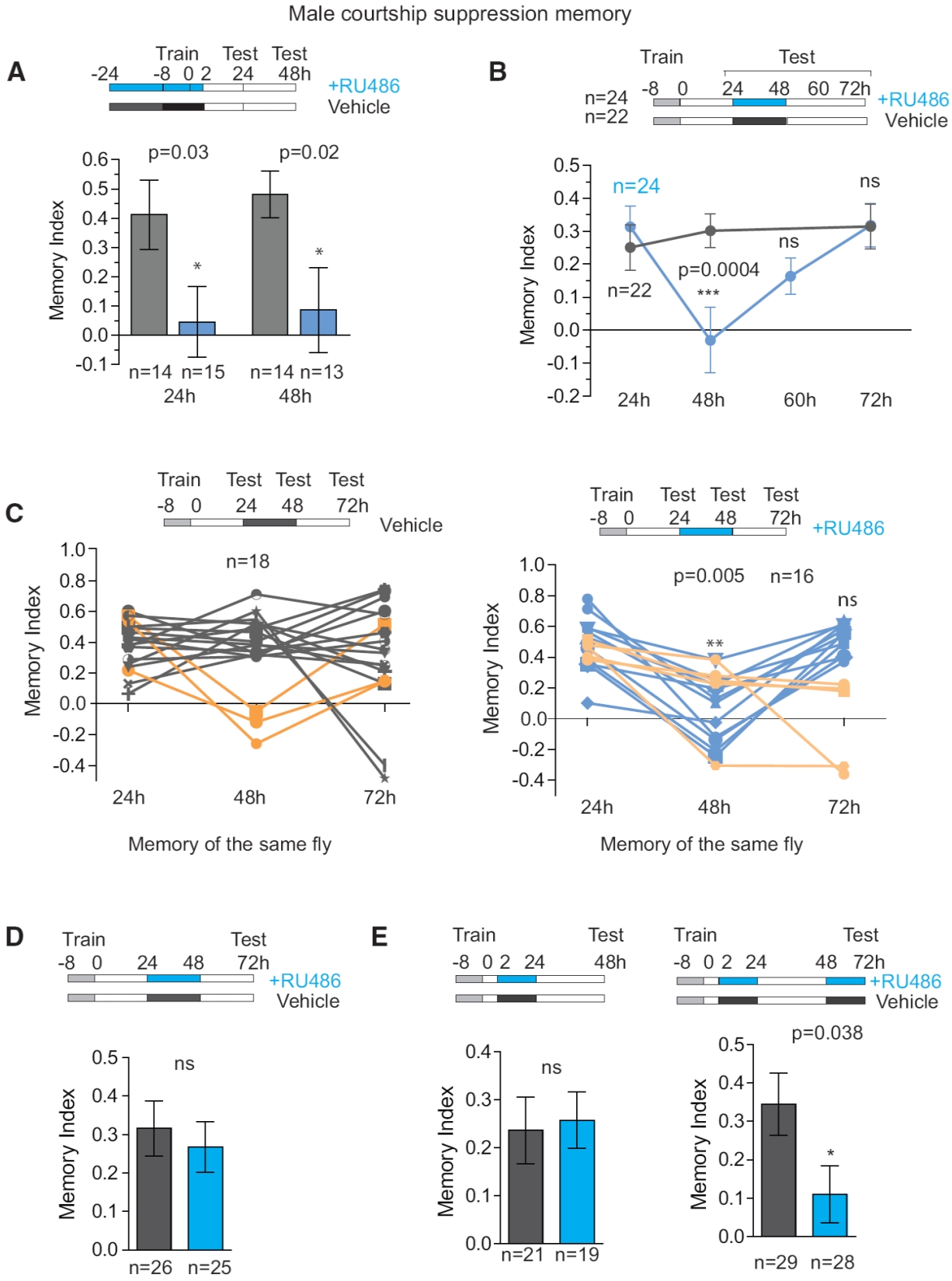


Figure 4.4 Orb2 activity is required for consolidation and maintenance of long-term memory in appetitive associative memory.

A) Schematic of the appetitive associative memory paradigm. One to two days old flies were starved for 18-22h and then trained to associate either MCH or OCT with 1M sucrose as reward. Following training flies were given either 1mM RU486 (blue) or vehicle (black) and then starved again before testing for their odor preference.
B) Feeding of RU486 before and any time after training reduces appetitive associative memory.
C) RU486 or TEV-protease does not interfere with long-term memory. Flies with indicated genotype were fed with RU486 (blue) or vehicle (black) for 24h post-training. Only in flies expressing both modified Orb2 and TEV protease significant loss in memory was observed.

Memory index for each group was plotted as mean ± SEM and statistical significance was determined by unpaired t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.


Male courtship suppression memory
A
Train
Test
Test
-24
-8
0
2
24
48h
+RU486
Vehicle
p=0.03
p=0.02
Memory Index
n=14
n=15
n=14
n=13
24h
48h
B
Train
Test
n=24
n=22
-8
0
24
48
60
72h
+RU486
Vehicle
n=24
ns
ns
p=0.0004
n=22
***
Memory Index
24h
48h
60h
72h
C
Train
Test
Test
Test
-8
0
24
48
72h
Vehicle
n=18
Memory Index
24h
48h
72h
Memory of the same fly
+RU486
p=0.005
n=16
ns
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Memory Index
24h
48h
72h
Memory of the same fly
D
Train
Test
-8
0
24
48
72h
+RU486
Vehicle
ns
Memory Index
n=26
n=25
E
Train
Test
-8
0
2
24
48h
ns
Memory Index
n=21
n=19
Train
Test
-8
0
2
24
48
72h
+RU486
Vehicle
p=0.038
Memory Index
*
n=29
n=28

Figure 4.5 Orb2 is required for storage, retrieval and recovery of memory in courtship suppression paradigm.

A) Reducing Orb2 during or immediately after training and providing only during recall is not sufficient for courtship-suppression memory. Flies were fed with RU486 (blue) or vehicle (black) 24 hr before, during, and 2 hr after training and then back to normal food for 24 hr or 48 hr before testing.

B) Once established, memory can recover if Orb2 is re-expressed. One day after training, flies were fed with RU486 or vehicle for 24 hr and then transferred back to normal food for 12 hr (60 hr) or 24 hr (72 hr) before testing. The same group of flies were trained and tested at 24 hr, 48 hr, 60 hr, and 72 hr posttraining.

C) Memory recovery in the same fly. Memory score of the same individual fly at 24 hr, 48 hr, and 72 hr. One day post-training, flies were fed with vehicle (left) or RU486 (Wright and Dyson). Feeding of RU486 or vehicle was restricted between 24- and 48-hr periods. Each line represents memory dynamics of individual fly at indicated time point. Black (vehicle) and blue (RU486) lines represent the main trend, and orange lines represent deviations from the main trend. (B) and (C) are independent experiments.

D) Memory recovery is independent of testing. Flies trained in the male courtship suppression paradigm were fed RU486 (blue) or vehicle (black) for 24 hr 1 day after training and then back to normal food for 24 hr before testing (white).

E) Recovered memory requires Orb2. Flies were fed with RU486 (blue) or vehicle (black) during two indicated periods of time: 2–24 hr (left) and 48–72 hr (Wright and Dyson) and memory was tested at 48 hr (left) and 72 hr (Wright and Dyson). The flies were rested for 2 hr before RU486 exposure to allow memory formation.

Memory index at each time point was plotted as mean ± SEM. Statistical significance was determined by unpaired t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Chapter 5. Modulation of aggregated Orb2 changes memory stability

***Acknowledgements**:* This part of work is collaborated with Consuelo Perez Sanchez, Yubai Zhao, Dr. Rubén Hervás, Dr. Amitabha Majumdar and Dr. Erica White-Grindley.

5.1 Objectives and rationale

The identification of an aggregation defective Orb2 mutant (Orb2F5Y) and the long-term memory deficiency in these flies (Majumdar et al., 2012) suggested a critical role of aggregated Orb2 in long-term memory. However, a mutation in the protein can also possibly affect other properties of the protein, such as protein-protein interaction and post-translational modification, in addition to aggregation. Therefore, other approaches to modulate Orb2 aggregation are necessary to unequivocally establish the causal link between Orb2 aggregation and memory stability. To this end, other members of the lab have identified 1) a protein network that controls Orb2 oligomerization through phosphorylation-dephosphorylation of Orb2A, 2) a small anti-amyloidogenic peptide polyglutamine-binding peptide 1 (QBP1) that blocks Orb2 oligomerization, and 3) a DNA-J family chaperone JJJ2 that facilitates Orb2 aggregation. These reagents allow me to address the necessity as well as sufficiency of aggregated Orb2 in memory consolidation. In this chapter, I have tested how these various reagents affect memory.

5.2 Tob Is Required for Long-Term Memory

Through proteomics analysis previous members of the lab had identified the Orb2 protein complex. Transducer of Erb2, Tob, is one of the strongest candidates that associate with both Orb2A and Orb2B (White-Grindley et al., 2014b). Moreover, Tob enhances Orb2 oligomerization

in two ways (Fig 5.1A): first it stabilizes Orb2A protein, which serves as seed for Orb2 amyloid formation; and second it recruits neuronal specific kinase, Lim Kinase, to promote Orb2 phosphorylation, a critical step for Orb2 conformation switch into oligomeric state. Because Orb2 oligomerization is important for long-term memory and Tob affects Orb2 oligomerization, I wondered whether Tob activity is important for long-term memory. To this end, I used the male courtship suppression paradigm in which a virgin male fly learns to suppress its courtship behavior upon repeated exposure to an unreceptive female (Hall, 1994). As I have tested for male courtship suppression memory Orb2 activity in the mushroom body γ-lobe neurons is important (Chapter 3), I knocked down Tob expression by expressing TobRNAi under mushroom body γ-lobe driver 201Y Gal4 (Zars et al., 2000b). I found that male flies expressing TobRNAi (201Y:Gal4-UAS-TobRNAi) in the γ-lobe showed courtship suppression in the short term (5 min), but the courtship suppression was lost when measured at 24 h or 48 h after training (Fig 5.1B). In contrast, flies harboring just the RNAi (UAS-Tob RNAi) or Gal4 (201Y:Gal4) had no impairment in courtship suppression 5 min or 24 to 48 h after training (Fig 5.1B). These results are consistent with the idea that Tob activity is important for long-term courtship suppression memory.

5.3 QBP1 Interferes with Memory Consolidation

Because of the structural similarity between amyloid-like aggregates adopted by Orb2 and the pathological amyloids that associated with fatal neurodegenerative disorders (Lansbury and Lashuel, 2006; Pepys, 2006), we and others seek to understand whether a peptide that inhibits amyloidogenesis has any effect on Orb2 aggregation and memory. One of the known anti-amyloidogenic peptide, polyglutamine-binding peptide 1 (QBP1) (Nagai et al., 2003; Nagai et al., 2000), has been shown to block Orb2 amyloidogenesis (Hervás et al., 2016). Since QBP1 inhibits

the transition from the monomeric state to a conformation that leads to amyloid formation, I tested the physiological consequences of QBP1 expression on memory consolidation. Using the Gal4-UAS system, the QBP1-cyan fluorescent protein (QBP1-CFP) or a control scrambled version of QBP1 (SCR-CFP), were expressed pan-neuronally (Nagai et al., 2003). Neither pan-neuronally expressed QBP1-CFP or SCR-CFP affect fly development, although normal courtship behavior was slightly dampened. Following training, both experimental flies (Elav-Gal4:UAS-QBP1-CFP or SCR-CFP) and the genetic controls (Elav-Gal4 and UAS-QBP1 or UAS-SCR) displayed a similar suppression of courtship immediately after training, suggesting that the expression of these peptides does not interfere with learning or short-term memory (Fig 5.2A). However, when measured after 24 h, the Elav-Gal4:UAS-QBP1 males displayed loss of long-term memory compared to the Elav-Gal4:UAS-SCR control flies (Fig 5.2A). To determine whether QBP1-mediated memory loss is independent of Orb2, I expressed QBP1 in Δ80QOrb2 flies that lack N-terminal Q-rich 80 amino acid residues of Orb2. The Δ80QOrb2 flies form short-term memory but no long-term memory (Keleman et al., 2007; Majumdar et al., 2012). Expression of QBP1 in Δ80QOrb2 had no additive effect in the loss of long-term memory (Fig 5.2A).

Since QBP1 is a low affinity peptide that can only block early stages of oligomerization but not already formed oligomer, I also investigated the consequence of transient expression of QBP1 in courtship suppression memory. To this end, I used RU486-inducible GeneSwitch Elav-Gal4 system and induced expression of the QBP1 peptide in the adult flies 24 h before training (Osterwalder et al., 2001). Transient expression of QBP1 had no effect on the long-term courtship suppression memory, consistent with the idea that QBP1 cannot interfere with pre-formed oligomers (Fig 5.2B). Finally, to determine whether chronic expression of QBP1 results in general disruption of the nervous system, I trained the ElavGal4-UASQBP1 flies in a heat-box paradigm.

In this operant conditioning paradigm, flies learn to avoid one side of an otherwise symmetrical chamber (Putz and Heisenberg, 2002). Intriguingly, the heat box paradigm produces robust short-term memory, but the memory does not persist beyond an hour or two. The memory curve of QBP1 was like that of wild type flies (Fig 5.2C), suggesting that QBP1 expression does not interfere with the animal's ability to form short-lived memories. Taken together, these results suggest that chronic QBP1 expression can interfere with some form of long-term memory and the effects of QBP1 in memory may be partly mediated through Orb2.

5.4 Expression of JJJ2 enhances memory

Mutations in Orb2, interfering Orb2 phosphorylation or peptide inhibitors that prevent Orb2 aggregation suggested that the conformational switch of Orb2 to the aggregated state is necessary for long-term memory (Hervás et al., 2016; Majumdar et al., 2012). However, the consequence of facilitation of Orb2 aggregation, if any, is still unknown. Chaperones are proteins that guide protein folding and maintain protein homeostasis (Kim et al., 2013). Previously we found that the Orb2A prion-like domain (Orb2AprD) can substitute the yeast Sup35 prion-like domain, and that the Orb2AprD-Sup35C fusion protein readily undergoes prion-like conversion in yeast (Hervás et al., 2016). Others have reported that yeast chaperones such as Hsp104 can influence aggregation of mammalian prion-like proteins (Cashikar et al., 2005; Vacher et al., 2005). Therefore we carried out an unbiased survey of the chaperones in yeast *S. cerevisiae* (Vacher et al., 2005) by knocking out or overexpression of individual chaperones in a background that allows for the score of prion-like conversion of an Orb2Aprd-Sup35C chimeric protein (Fig 5.3A&B). Both the deletion and overexpression screen identified JJJ2, a lowly expressed (~200 molecules/cell, Yeast Genome Database) DNA-J domain containing protein in Hsp40 family (Gillies et al., 2012) that surprisingly facilitates the aggregation of Orb2AprD-Sup35C protein in

yeast cells (Fig 5.3A&B). We then introduced JJJ2 in the *Drosophila* S2 cells and co-expressed with Orb2. Consistent with the yeast screen, JJJ2 enhances Orb2 aggregation as shown an increase in Orb2 oligomer in 7% SDS-PAGE as well as a smear tail in 1.5% SDS-Agarose, indicative of higher molecular assembly (Fig 5.3C). In contrast, JJJ2 mutants lacking the chaperone activating domain (JJJ2ΔJ) or the J domain only did not have any effect on Orb2 protein (Fig 5.3C). Interestingly, JJJ2 has a more profound effect on Orb2A protein than Orb2B: not only oligomeric Orb2A is increased, monomeric Orb2A is shifted to higher molecular weight (Fig 5.3C), suggesting JJJ2 induces or stabilizes some intermediate protein conformation of Orb2A.

We also tested whether JJJ2 can functionally affect Orb2-mediated translation by an *in vitro* translation assay established in our lab (Khan et al., 2015). This assay takes advantage of the fly embryo extract that contains all the translation machinery but lacks the Orb2A protein. Previous studies showed that the addition of Orb2A *in vitro* can transform endogenous Orb2B from translation repressor to activator (Khan et al., 2015). Addition of Orb2A and JJJ2 each enhances translation of a reporter construct; combination of both resulted in an additive enhancement in translation (Fig 5.3D). JJJ2 lacking the J domain (JJJ2ΔJ) or just the J-domain had no effect on translation (Fig 5.3D). Importantly, JJJ2 induced enhancement of translation is dependent on Orb2, since adding JJJ2 to an *orb2* null embryo extract had no effect on translation (Fig 5.3D). These results indicate JJJ2 enhances functional Orb2 aggregation.

Since JJJ2 can enhance aggregation of Orb2 as well as Orb2-mediated translation, next we sought to determine whether JJJ2 influences long-term memory. To this end, we generated transgenic flies expressing HA-tagged JJJ2 under the Gal4-UAS system (Brand and Perrimon, 1993). Flies carrying JJJ2 as a transgene were similar to wild type flies in fecundity, lifespan, locomotion and sensory perception. Normally in the male courtship suppression memory paradigm

three 2h training sessions (Fig 3.3A) are required to generate long-term memory; one training session of 2 hours leads to no or low memory in wild type flies (Fig 5.4A) (Majumdar et al., 2012; McBride et al., 1999). Unexpectedly, we observed that flies harboring a single copy of JJJ2 transgene (UAS-JJJ2-HA>attp40), even in the absence of any Gal4 driver, formed significantly higher memory following one or two training sessions compared to wild type flies (Fig 5.4A) and the memory persisted over days (Fig 5.4B). Immunopurification revealed low level of JJJ2HA protein expression in the UAS-JJJ2HA>attp40 adult fly head (Fig 5.4C). The following controls suggest memory enhancement is not an artifact of insertion at attp40 site in 2nd chromosome and requires full length JJJ2 protein expression (Fig 5.4D&E): there was no increase in memory in flies harboring 1) JJJ2 with a single nucleotide frameshift after the 5th amino acids that introduces a stop codon in all three reading frames (UAS-JJJ2FS>attp40); 2) JJJ2 lacking the DNA-J domain (UAS-JJJ2ΔJ>attP40) (Kesti et al., 2004); 3) only the 66 amino-acid DNA-J domain (UAS-J domain>attP40); 4) the close family member JJJ3 (UAS-JJJ3HA>attp40); or 5) full length wild type JJJ2 at attp2 site on 3rd chromosome (UAS-JJJ2HA>attp2) that did not show any detectable expression (Fig 5.4C). The UAS-JJJ2>attp40 also failed to improve memory of Orb2Δ80Q flies lacking the part of the prion-like domain (Fig 5.4E) (Keleman et al., 2007), indicating JJJ2-mediated memory improvement either requires Orb2 or can't circumvent the Orb2 deficiency. Finally, to determine the effect of Gal4-driven expression, UAS-JJJ2>attp40 flies were crossed to 201Y-Gal4 to drive expression in the mushroom body. Crossing to 201Y resulted in an increase in memory following 1X training only in JJJ2, but not in the J domain lacking mutant (Fig 5.4F). Taken together, our observations suggest low amount of JJJ2 lowers the threshold for consolidation of long-term memory. Interestingly, higher level of JJJ2 induced by stronger Gal4 does not necessarily generate better memory under suboptimal training. This is consistent with the

chaperone working model that only small amount of chaperone is needed for the conformation switch of the client protein. Whether there is a fly Hsp40 chaperone that, like JJJ2, regulates specifically Orb2 conformation is of interest.

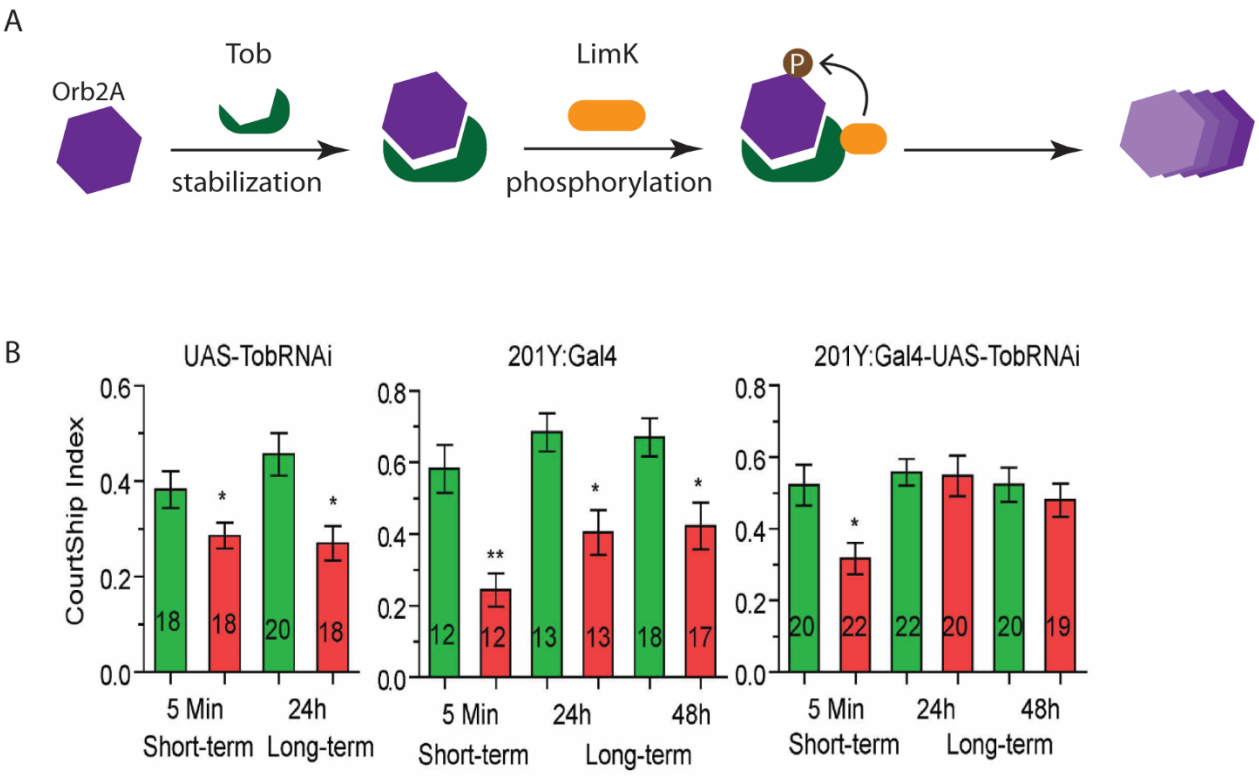

Figure 5.1 Tob Is Required for Long-Term Memory.

A) Model of Tob mediated Orb2 oligomerization. Tob binds and stabilizes Orb2A. Tob then recruits activated Lim kinase to the complex, which phosphorylates Orb2A. Phosphorylation of Orb2A leads to further stabilization and conformational change that results in oligomerization.

B) Reduction of Tob in mushroom body γ lobe (201Y:Gal4-UAS-TobRNAi) impairs male courtship suppression memory at 24 h and 48 h, as no significant difference in courtship activity is observed between untrained (green) and trained (red) group. The 5 min memory remains intact. The heterozygotes control flies for TobRNAi and 201Y:Gal4 show memory at all time points. The numbers indicate number of animals examined in each experimental group. The plots indicate mean courtship index ± SEM. Statistical significance was determined by unpaired t-test. *$p < 0.05$.

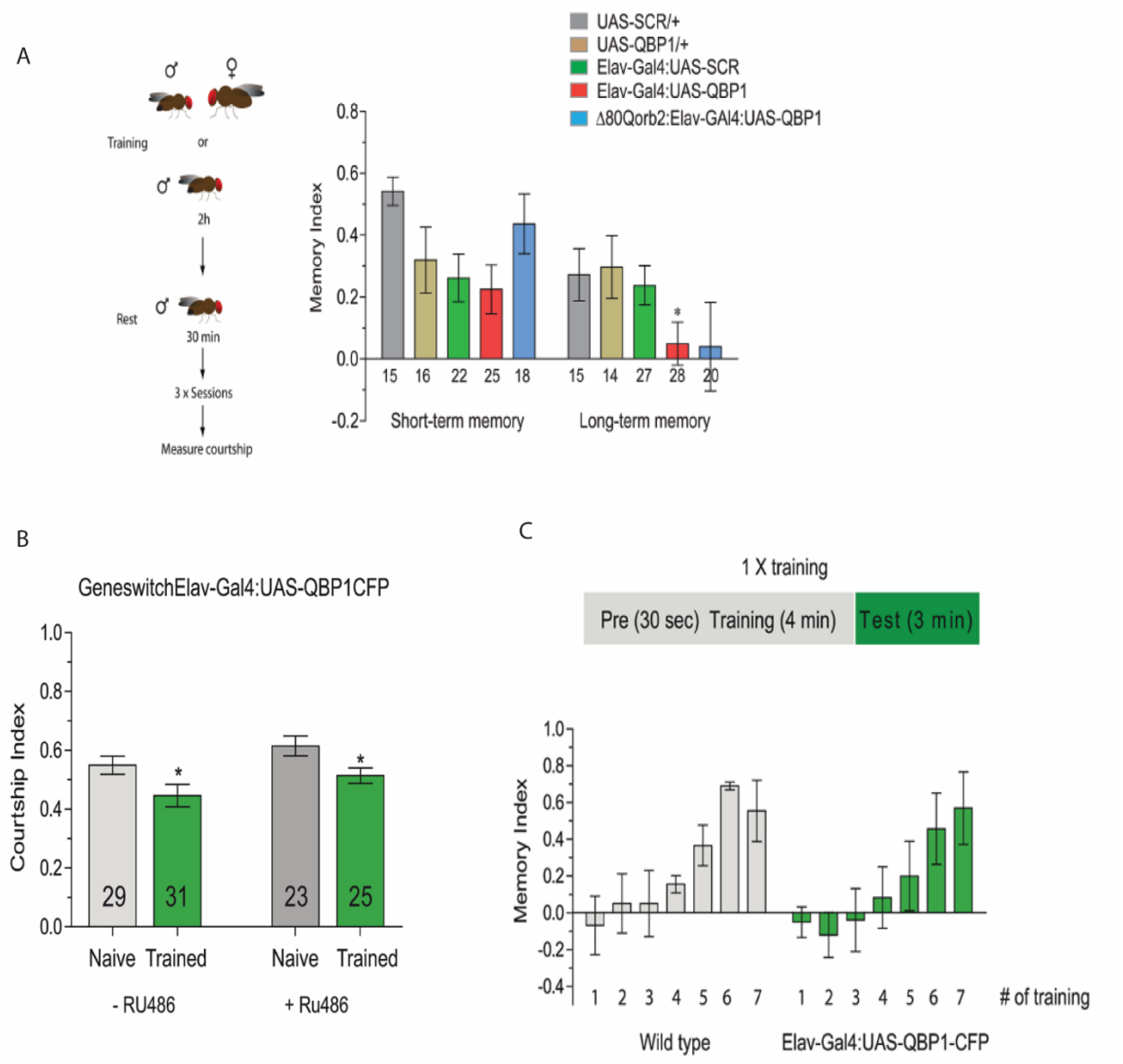

Figure 5.2 QBP1 interferes with Orb2-mediated memory consolidation *in vivo*.

A) Pan-neuronal expression of QBP1 disrupts long-term male courtship suppression memory, while it does not interfere with short-term memory. Expression of QBP1 in the Δ80Q orb2 mutant background did not have any additive effect on long-term memory. The data are represented as the mean ± SEM. We assumed statistical significance at *$p < 0.05$ (One-way ANOVA).

B) Transient expression of QBP1 does not interfere with long-term memory. The QBP1 peptide was expressed in the adult nervous system 1 d prior to the behavioral training using the RU486-inducible GeneSwitch-Gal4 system. The 4-5 days old flies were fed with 1 mM

RU486 for 24 h to induce the expression of QBP1. Following drug feeding the flies were trained in the male courtship suppression paradigm and memory was measured at 24 h. Both control (-RU486) and experimental groups showed significant courtship suppression at 24 h, suggesting that long-term memory was not affected by the transient expression of QBP1. The number of flies tested in various groups are indicated. *$p < 0.05$ (unpaired two-tailed Student's *t* test).

C) QBP1 expression does not interfere with short-term avoidance memory. **Top**: schematic of the single training protocol in the heat box operant conditioning paradigm. In this operant conditioning paradigm, individual flies were conditioned to avoid one side of a uniform chamber. Each time the fly enters the predetermined "punish" side of the chamber, the temperature of the entire chamber is heated to 37°C and the temperature is brought back to 24°C when the fly moves to the other "unpunished" side. Following training, the flies learn to avoid the punish side even in the absence of punishment. Memory is measured as the duration of the preferred response for unpunished side. Flies are either trained in a single trial protocol or multiple trial protocol where the single training protocol is repeated successively the indicted times. **Bottom**: the avoidance index after each training session. With repeated training, the avoidance index increases, and after six training sessions the performance plateaus. There was no significant difference in the performance between wild type flies and flies expressing QBP1 chronically in the nervous system.

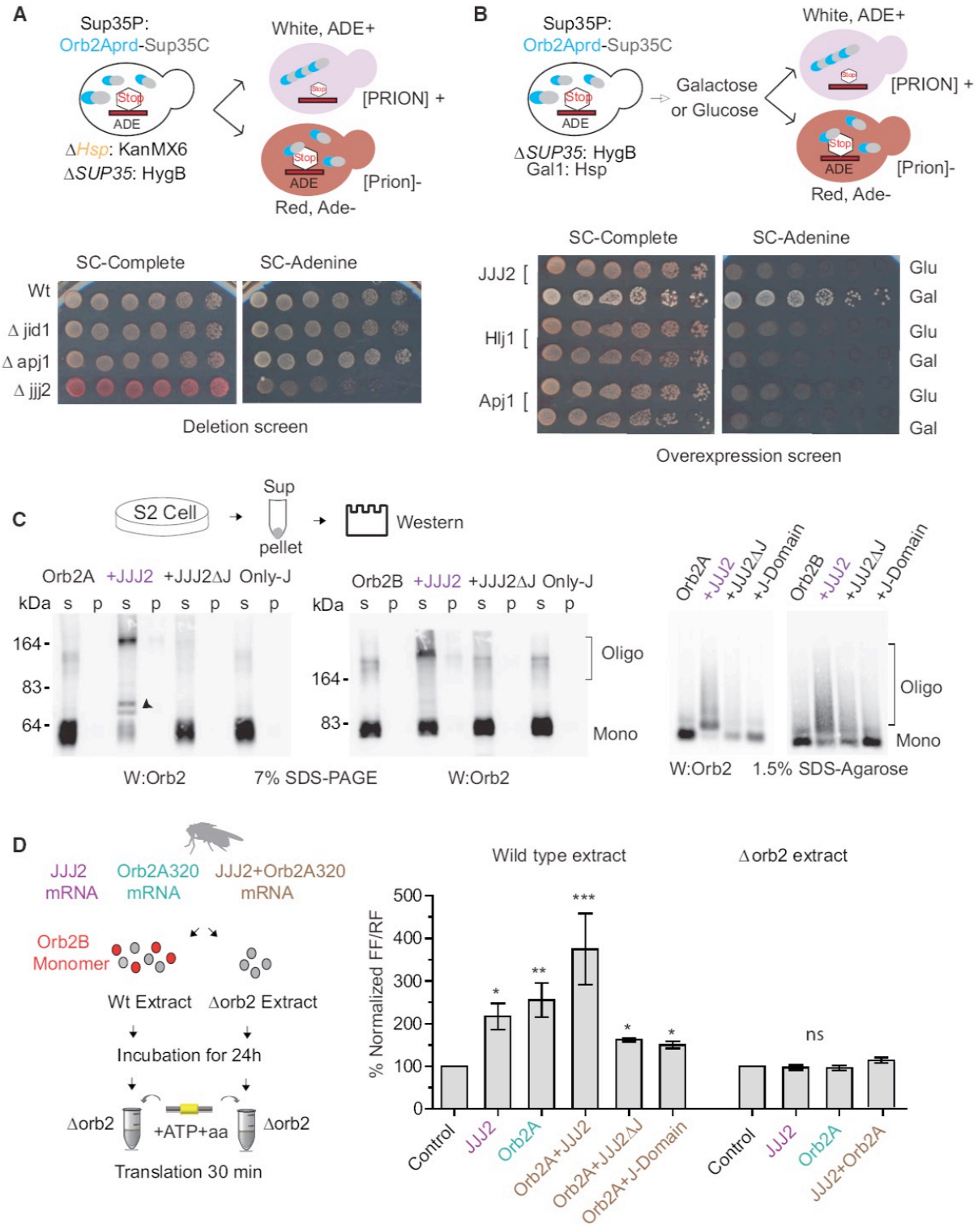

Figure 5.3 Yeast Hsp40 family protein JJJ2 enhances functional Orb2-aggregates in heterologous system.

A) Top panel: Schematic of yeast Hsp deletion screen. Bottom panel: In JJJ2 deletion background, but not in wild type or other Hsps such as Jid1, or Apj1 deletion background colonies appear red in rich media and cannot grow on –adenine media.

B) Top panel: Schematic of yeast Hsp overexpression screen. The yeast chaperons were ectopically expressed under the inducible Gal promoter in Orb2AprD-Sup35C background. Same strains grown in Glucose serve as a control. Bottom panel: Overexpression of JJJ2, but not other Hsps such as Hlj1 or Apj1 facilitates growth in –adenine media.

C) JJJ2 induces aggregation of Orb2A and Orb2B in S2 cells. The cell lysate was run in 7% SDS-PAGE or 1.5% SDS-Agarose gel to reveal monomeric and oligomeric Orb2. JJJ2ΔJ (JJJ2 protein lacking the N-terminal 66 amino acids encompassing the DNA-J domain) and only-J (N-terminal 66 amino acids of JJJ2 containing the DNA-J domain) serves as control.

D) JJJ2 enhances Orb2 aggregation-dependent translation. Left panel: Schematic of the *in vitro* translation assay using *Drosophila* embryo extract. JJJ2 mRNA (purple), Orb2A320 mRNA (green), and JJJ2 + Orb2A320 mRNA (brown) were translated *in vitro* then incubated with wild type or *Δorb2* embryo extract as control for 24h to allow for conformational alteration of monomeric Orb2B protein in the embryo extract. The mix was then added into *Δorb2* embryo extract containing reporter mRNAs to measure translation. Values of fire fly luciferase/renilla luciferase of each group were normalized to control mRNA group and each experiment is comprised of three independent repeats. Statistical significance was determined using One-way ANOVA for multiple groups and data are expressed as mean ± SEM. The * indicates $p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

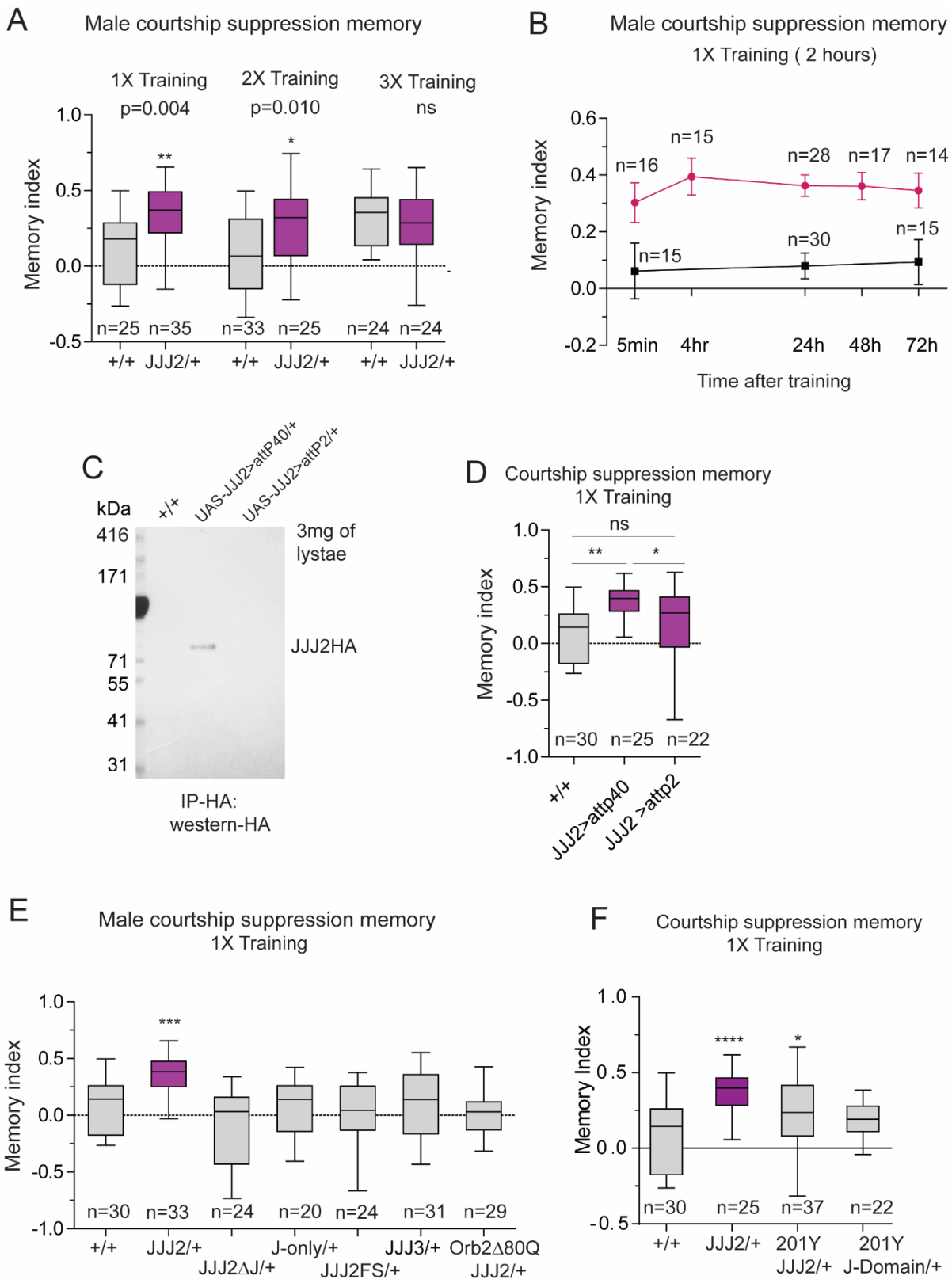

Figure 5.4 JJJ2 enhances long-term courtship suppression memory.

A) JJJ2-expressing flies forms better long-term memory after suboptimal training. Wild type (gray) and UAS-JJJ2-HA>attP40 (purple) flies were subjected to 1X, 2X or 3X male courtship suppression training sessions and memory was tested after 24h hours.
B) Wild-type (gray) and UAS-JJJ2-HA>attP40 (purple) flies were subjected to 1x male courtship-suppression training session, and memory was tested at indicated time after training.
C) Leaky expression of JJJ2HA protein in UAS-JJJ2>attP40 fly head. JJJ2HA was immunoprecipitated with anti-HA from 3mg of fly head lysate of wild type, UAS-JJJ2>attP40 and UAS-JJJ2>attP2 flies and western blotted with anti-HA antibody.
D) Only in UAS-JJJ2>attP40 but not in UAS-JJJ2>attP2 flies significant increase in 24h courtship suppression memory was observed following 1X training.
E) Memory enhancement following 1X training requires full length JJJ2 and is Orb2-dependent.
F) Expression of JJJ2 (UAS-JJJ2>attP40) but not J-Domain in mushroom body neurons under 201YGal4 drivers enhances courtship suppression memory. Interestingly, although the memory was enhanced it was less compared to just UAS-JJJ2>attp40 flies suggesting more of JJJ2 is not necessarily conducive to better memory and integration of JJJ2 at attP40 site serendipitously provided the appropriate amount to aid memory formation.

Data are expressed as mean ± SEM and statistical significance was determined using One-way ANOVA for multiple groups, unpaired t-test for two groups. *$p<0.05$, ** $p<0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

Chapter 6. Oligomerization of Orb2 as a biochemical engram of long-term memory

6.1 Objectives and rationale

The observations that Orb2 is required to encode and retrieve long-term memory (Chapter 4), interfering with Orb2 aggregation inhibits memory (Chapter 5), and that facilitation of Orb2 aggregation enhances memory (Chapter 5), suggested that Orb2 aggregation may be specific process that is only engaged during long-lasting memory formation. If that is true then the neuron in which Orb2 aggregates are likely to be the site of memory storage. Therefore, I set out to visualize activity-dependent aggregation of Orb2 in the adult brain (observing the engram).

6.2 Reconstitution of TEV-protease activity in Orb2-aggregation-dependent manner

There are various methods to assay protein aggregation in the cells and other lab members have explored them extensively such as immunostaining or use of fluorescence protein tag. However, as Orb2 is low abundant in the brain, these methods did not give high signal/noise ratio. Forster Resonance Energy Transfer (FRET) based assay is dependent-on energy transfer between two chromophores when they are in the range of 1-10nm. FRET efficiency is also dependent on the orientation of the packed molecules and we found that the packing of Orb2 oligomers is not optimal for FRET. Therefore I sought for techniques that can amplify the signal from aggregated Orb2.

In TEV protease reconstitution system (Wehr et al., 2006) the TEV protease is split into N-terminal (TEVN) and C-terminal (TEVC) halves and fused with interacting proteins. Only direct protein-protein interaction reconstitutes TEV protease activity and TEV enzymatic activity indicates the extent of interaction. Since TEV protease by itself does not impair memory (Chapter 4) and the TEV protease is a small protein (27kD) and when split, makes a 13kD small tag, I

decided to use the split-TEV protease system to study Orb2 aggregation. To this end, chimeric Orb2 proteins carrying TEVN and TEVC at the C-terminal end of Orb2 (Orb2-TEVN and Orb2-TEVC) were expressed first in *Drosophila* S2 cells (Fig 6.1A). The co-expression of Orb2-TEVN and Orb2-TEVC reconstitutes TEV–protease activity and the reconstituted TEV-protease can act on a variety of substrates bearing a TEV protease site (TevS: ENLYFQ/S) (Fig 6.2B). The substrates were cleaved only when both Orb2-TEVN and Orb2-TEVC were present, while expression of them separately, or TEVN+TEVC without attachment to Orb2, did not have measurable enzymatic activity. Thus, I concluded that homo-interaction between Orb2 proteins can reconstitute functional TEV protease activity.

To determine whether this system could be used in the adult fly brain, the endogenous Orb2 was replaced with a genomic fragment of one copy of Orb2-TEVN and one copy of Orb2-TEVC (Fig 6.1A). The modified Orb2 genomic fragment rescues both developmental and memory deficit of Orb2 mutant, suggesting the small split TEV-protease tag does not interfere with protein function. To measure Orb2-splitTEV reconstitution *in vivo* I developed a destabilized-luciferase reporter (Sellmyer et al., 2009), in which luciferase is fused to FKBP destabilizing domain (DD) with a TEV protease site in between (Fig 6.1A). Expression of the FKBP-DD-Luciferase in the adult head neurons led to a very low activity (3 fold less) compare to luciferase alone and co-expression of TEV protease increases the luciferase activity to the level similar to the wild-type luciferase (Fig 6.1C). After verifying the FKBP-DD-Luciferase reporter, I expressed it pan-neuronally in the Orb2-TEVN/Orb2-TEVC genomic substitution flies. Orb2-mediated reconstitution of TEV protease (Orb2-TEVN + Orb2-TEVC) resulted in a significant increase of luciferase activity in the brain compared to the flies carrying only one half of the TEV protease (Orb2-TEVN) (Fig 6.1D). Orb2A isoform is low abundant in the fly head but critical for Orb2

oligomerization. The increase in luciferase activity was also observed in the Orb2A deletion flies (Orb2ΔA-TEVN/Orb2ΔA-TEVC), however was not statistically significant compared to the control (Orb2-TEVN) (Fig 6.1D), suggesting some basal Orb2 oligomerization is mediated by Orb2B. Furthermore, tyramine, which enhances Orb2 aggregation (Majumdar et al., 2012), also significantly increased luciferase activity only in Orb2-TEVN/Orb2-TEVC flies but not in aggregation defective Orb2ΔA-TEVN/Orb2ΔA-TEVC flies (Fig 6.1E). These observations suggest that Orb2 can reconstitute TEV protease activity *in vitro* and *in vivo* and TEV-protease activity can be a proxy for Orb2 aggregation.

6.3 Extent of Orb2 aggregation in the γ-lobe of mushroom body is predictive of memory strength

The luciferase assay gave a sensitive readout of Orb2 aggregation in the adult fly head. However, from the luciferase based assay it's not clear in how many neurons and where Orb2 aggregates. Therefore, to visualize and to quantify Orb2-splitTEV reconstitution *in vivo* I developed a GFP-based TEV-protease reporter, GFP-dark (Fig 6.1A). In GFP-dark a small quenching peptide, which diminishes GFP fluorescence (Nicholls et al., 2011), is attached to the C-terminus end with an intervening TEV-cleavage site; expression of TEV, and thus removal of the quenching peptide, resulted in significant increase in GFP fluorescence in different subpopulation of fly head neurons (Fig 6.2A-D), while expression of the TEV protease does not have measurable effect on GFP without any quencher (Fig 6.2D). Importantly, both GFP-dark and GFP have half-lives of ~5 hours in the fly brain allowing for a more dynamic readout of TEV protease activity. I further expressed the GFP-dark reporter pan-neuronally in the Orb2-TEVN/Orb2-TEVC flies. There was a higher GFP signal in the mushroom body γ lobe, but not in

α/ α' and β/ β' lobes, in the Orb2-TEVN/Orb2-TEVC flies compared to controls (Orb2-TEVN) (Fig 6.2E), whereas in the α/ α’ and β/ β’ lobes there is no significant difference in GFP intensity. These observations suggest Orb2 prone to aggregate in the mushroom body γ neurons and are consistent with other study that Orb2 functions in the mushroom body γ lobe for memory beyond a day (Keleman et al., 2007).

To determine the relationship between Orb2 aggregation and memory strength (if any), I sought for a memory paradigm that can assay a wide range of memory strength. In male courtship suppression paradigm a male fly learns to suppress its courtship behavior for days after repeated rejection by an unreceptive female (Fig 3.3A) (McBride et al., 1999; Siegel and Hall, 1979). This paradigm serves the purpose because it is a single fly memory assay, which allows for survey of population variation in memory stability; and memory is scored linearly instead of binarily, providing a spectrum of memory strength. I trained Orb2-TEVN/Orb2-TEVC flies pan-neuronally expressing GFP-dark and tested memory after a day. Immediately after testing the trained and mock trained fly brains were dissected and imaged (Fig 6.3A). Each fly has its own courtship suppression index (indicative of memory strength: the higher the suppression, the stronger the memory) and the corresponding image. The images were then numbered in a randomized order and analyzed by Dr. Brian Slaughter blindly. Normalized GFP signal in the γ lobe was calculated for each image. Finally, for each fly the courtship suppression index and the matching normalized GFP signal were plotted in an x-y plot. I observed a significant positive correlation ($p=0.0014$) between courtship suppression index and GFP fluorescence in the γ lobe of trained (Fig 6.3B-C) flies. No positive correlation was observed in the mock trained group (Fig 6.4A) as well as in a short-training protocol, which does not produce long-term memory (Fig 6.4B). These results suggested the positive correlation between Orb2 aggregation and memory strength is training

dependent. Furthermore, no positive correlation was observed in: i), flies expressing only one half of TEV (ElavGal4::UAS-GFP-dark; Orb2-TEVN/Orb2) (Fig 6.4C&D); or ii), flies expressing both halves of TEV but a GFP reporter that does not depend on TEV-protease activity (ElavGal4::UAS-GFP; Orb2-TEVN/Orb2-TEVC) (Fig 6.4E). These results suggest the correlation I observed is specific to training that induces long-term memory, is not due to expression of half TEV or fluctuation of GPF signal with memory. Moreover, a Monte Carlo simulation indicated that the correlation is significantly higher than expected by random chance (Fig 6.4F). Taken together these results suggest that Orb2-aggregation in the γ-lobe neurons is indeed predictive of the memory strength.

The positive correlation between Orb2-aggregation and memory of specific experience is somewhat surprising considering memory of past experiences and the uncertainty in behavioral manifestation of memory-flies may have memory but decide to act differently or by chance display a behavior that is consistent with memory-driven behavior. In spite of these "noise", significant positive correlation with long-term memory suggests, at least in *Drosophila*, Orb2 aggregation is likely a rare process engaged only when an animal forms long-lasting memory. What confers such specificity to this molecular process is of particular interest.

6.4 Artificially activating neurons in which Orb2 oligomerizes can retrieve a memory.

Since Orb2 aggregation can be a readout of memory strength, next I asked are the neurons in which Orb2 aggregates during memory encoding also recruited during memory retrieval. This requires marking the neurons in which Orb2 aggregates during memory encoding and activate or silence the same neuronal ensemble during memory retrieval. To this end, I coupled Orb2 mediated

TEV reconstitution activity to transcription of new genes such as light activated channelrhodopsin or light inhibited halorhodopsin to activate or silence neurons respectively (Fig 6.5A). I fused the Gal4 transcription factor to one half of the splitTEV linked by a TEV protease cleavage site (Orb2-TEVN-TevS-Gal4) and co-express this construct with Orb2-TEVC in S2 cell. TEV reconstitution in this scenario will cleave the Gal4. The free Gal4 then moves to the nucleus, targeting the UAS element and driving transcription of downstream genes. I used UAS-TdTomato as a reporter in S2 cell (Fig 6.5B). Co-expression Orb2-TEVN-TevS-Gal4 and Orb2-TEVC leads to significant increase of the cells expression TdTomato, while expressing the Orb2-TEVN-TevS-Gal4 alone shows undetectable signal (Fig 6.5B). These results suggest first the Orb2-TEVN-TevS-Gal4 restricts the Gal4 in the cytoplasm therefore maintains low transcriptional activity; and second TEV protease activity reconstituted by Orb2 aggregation frees Gal4 and allows transcription of UAS reporter.

To test whether this system works in the fly head, I substituted endogenous Orb2 with one copy of Orb2-TEVN-TevS-Gal4 and the other copy of Orb2-TEVC. In this fly, I introduced various UAS reporter (Fig 6.5A): 1) UAS-CD8GFP to visualize the neurons; 2) UAS-ReaChR, a red-shifted variant of channelrhodopsin that allows deep penetration of optic stimulation (Lin et al., 2013); 3) UAS-Jaws, a red-shifted halorhodopsin (Chuong et al., 2014). Training of flies carrying the UAS-CD8GFP labels a small number of mushroom body neurons when examined 24h after training (Fig 6.5C&D). Detection of the GFP positive neurons in the mushroom body is rare event and the expression pattern various from fly to fly (Fig 6.5D), suggesting either not enough GFP is made or the memory trace is sparse within the mushroom body neurons, which has been reported as a feature of mushroom body (Honegger et al., 2011) and mammalian piriform cortex (Stettler and Axel, 2009).

Next I tested whether I can activate these neurons by a red-shifted channelrhodopsin UAS-ReaChR and detect any behavioral consequence. In courtship suppression memory paradigm a male fly is exposed to the fresh mated unreceptive female and eventually learns to suppress its courtship towards another mated female when tested. However, if presenting the trained male to a virgin female, the male will become active in courtship again because the cues from virgin and mated females are different (Fig 6.5C). I then trained the males and allowed Orb2 to reconstitute TEV protease activity and express ReaChR in the relevant neurons. During test, instead of using unreceptive female, I used a decapitated virgin female (to reduce the copulation rate and keep male flies consistently motivated) (Fig 6.5C). When the red light is off, both trained and untrained flies court with decapitated virgin females vigorously, suggesting the trained flies do not associate rejection to the virgin female (Fig 6.5E upper panel). When the red light is simultaneously flashing during test to activate ReaChR, the trained flies show reduced courtship towards the virgin female (Fig 6.5E upper panel). Flashing of the light does not have any effect on the courtship of untrained flies. These observations suggest that during memory encoding, Orb2 oligomerizes in a small population of neurons; activating these neurons during test is sufficient to elicit behavioral manifested memory retrieval (Fig 6.5E bottom panel).

Unfortunately, my attempt to silence these neurons with Halorhodopsin failed likely due to inefficient expression or activation of the channel. Nonetheless, my observations connect the biochemical engram to the circuit engram of a long-term memory.

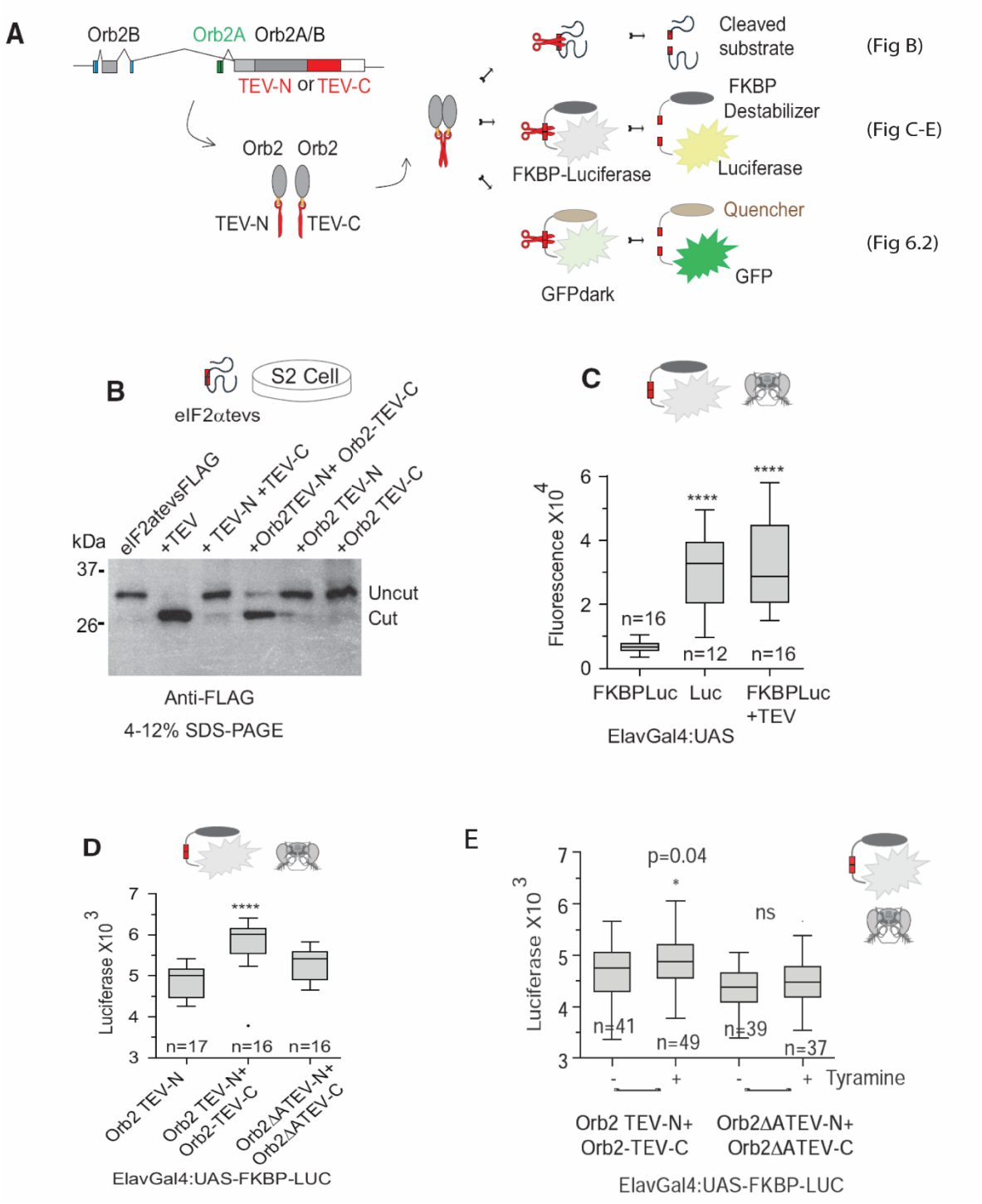

Figure 6.1 Orb2 aggregation reconstitutes TEV protease activity.

A) Schematic of Orb2 aggregation-dependent TEV protease reconstitution assay. Endogenous Orb2 is replaced by one copy of Orb2-TEVN and one copy of Orb2-TEVC genomic fragment. Different proteins bearing TEV protease recognition sequence (TevS) were tested.

The FKBP-DD-luciferase reporter is comprised of FKBP destabilize domain fused to firefly luciferase (dsLuciferase) with TevS in between. The reporter GFP-dark is comprised of a GFP attached to a quenching peptide with TevS in between.

B) FLAG tagged eIF2α-TevS (left) was expressed in S2 cells with the indicated construct. Western blot was probed for FLAG to detect uncut and cut fragments of eIF2α. Only Orb2TEV-N + Orb2TEV-C combination resulted in cleavage as efficient as fulllength TEV.

C) FKBP-DD-Luciferase or Luciferase was expressed panneuronally using ElavGal4. Co-expression of TEV protease with FKBP-DD-Luciferase resulted in significantly more luciferase activity compared to FKBP-DD-Luciferase only.

D) Reconstitution of TEV-protease activity in the adult fly head. FKBP-DD-Luciferase was expressed pan-neuronally on the genetic background of Orb2-TEVN/+, Orb2-TEVN/Orb2-TEVC or Orb2ΔA-TEVN/Orb2ΔA-TEVC. Statistical significance was determined using One-way ANOVA.

E) Tyramine stimulation increase split-TEV protease activity. FKBP-DD-Luciferase was expressed pan-neuronally in Orb2-TEVN/Orb2-TEVC or Orb2ΔA-TEVN/Orb2ΔA-TEVC flies. 10mM tyramine stimulation significantly increased luciferase activity only in Orb2-TEVN/Orb2-TEVC flies. Unpaired t-test was used to compare the effect of tyramine.

*$p < 0.05$, **$p < 0.01$, ***$p<0.001$, and ****$p<0.0001$.

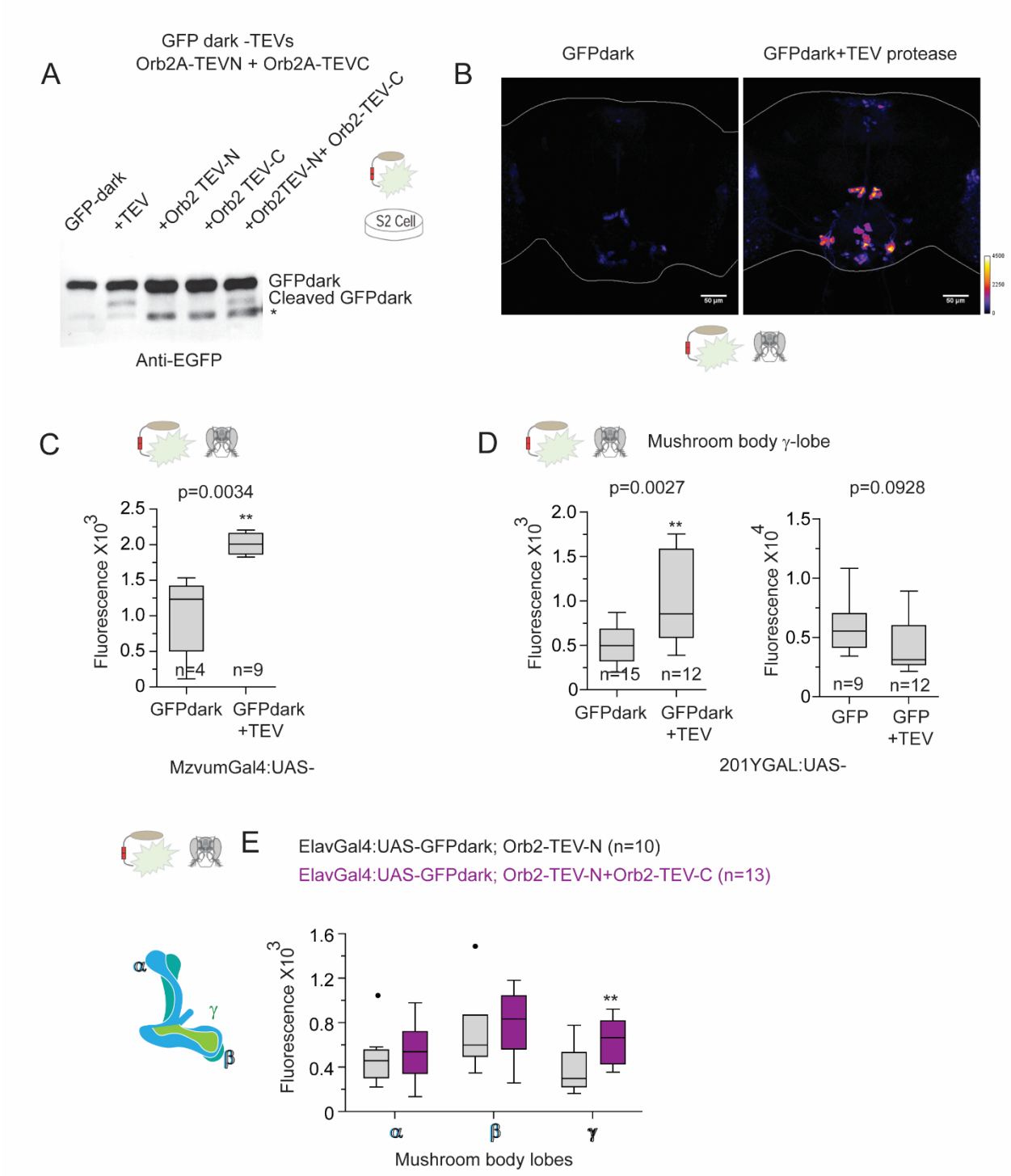

Figure 6.2 GFPdark as a reporter of Orb2 aggregation reconstitutes TEV protease activity *in vivo*.

A) GFP with a quenching peptide linked by a TEV protease recognition sequence (GFP-dark) was expressed in S2 cells. Co-expression of TEV protease or Orb2-TEVN/Orb2-TEVC resulted in

cleavage of GFP-dark. We observed that a small fraction of the GFP-dark is cleaved by the protease. The relative inefficiency worked in our favor because it reduced the noise in the system. Importantly the wildtype protease activity and the reconstituted protease activity were very similar for this substrate.

B) TEV-protease enhances fluorescence from GFP-dark reporter. GFP-dark was expressed in adult fly neurons without or with TEV protease.

C) Quantification of fluorescence intensity of the same set of neurons in (B).

D) Fluorescence intensity from the GFP-dark reporter but not from GFP reporter is enhanced upon expression of TEV-protease in the mushroom body neurons.

E) Fluorescence intensity of the GFP-dark reporter is significantly higher in the mushroom body γ-lobe neuron in Orb2-TEVN/Orb2TEVC flies than in Orb2-TEVN/+ flies.

Unpaired t-test was used to compare between groups. *$p < 0.05$, **$p < 0.01$.

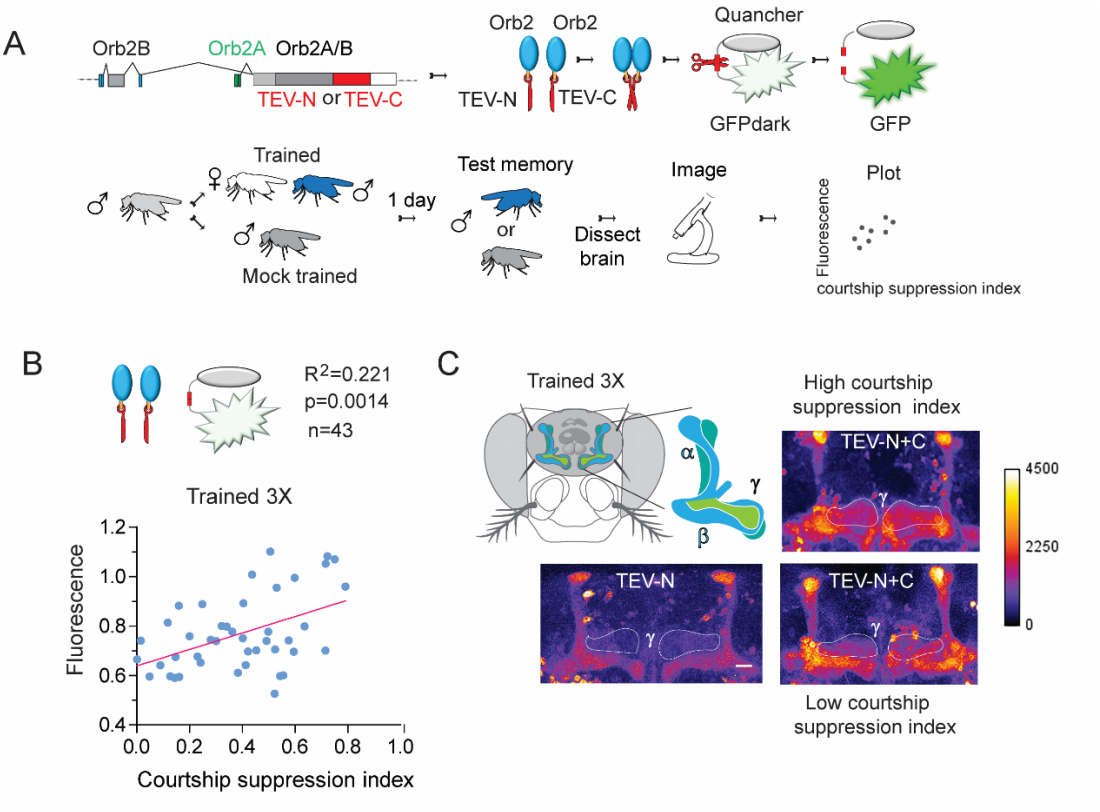

Figure 6.3 Orb2 aggregation in the γ-lobe of mushroom body is predictive of memory strength.

A) Schematic of Orb2 aggregation-dependent TEV-protease reconstitution assay. Endogenous Orb2 is replaced by one copy of Orb2-TEVN and one copy of Orb2-TEVC genomic fragment. The reporter GFP-dark is comprised of a GFP attached to a quenching peptide with TEV-protease recognition sequence in between. Trained or mock trained male flies were tested for memory at 24h and immediately after testing brains were dissected and imaged.

B) In 3X trained flies (ElavGal4::UAS-GFPdark; Orb2-TEVN/Orb2-TEVC) memory is positively correlated with GFP intensity in γ lobe.

C) Heat map images represent GFP signals in the mushroom body region of the following groups: Orb2-TEVN/Orb2 (bottom left), Orb2-TEVN/Orb2-TEVC high courtship suppression (upper right) and low courtship suppression (bottom right). The γ-lobe region is outlined. Scale bar: 20um.

Linear regression was used to analysis the correlation between courtship suppression index and GFP intensity. Only the positive courtship suppression index was plotted.

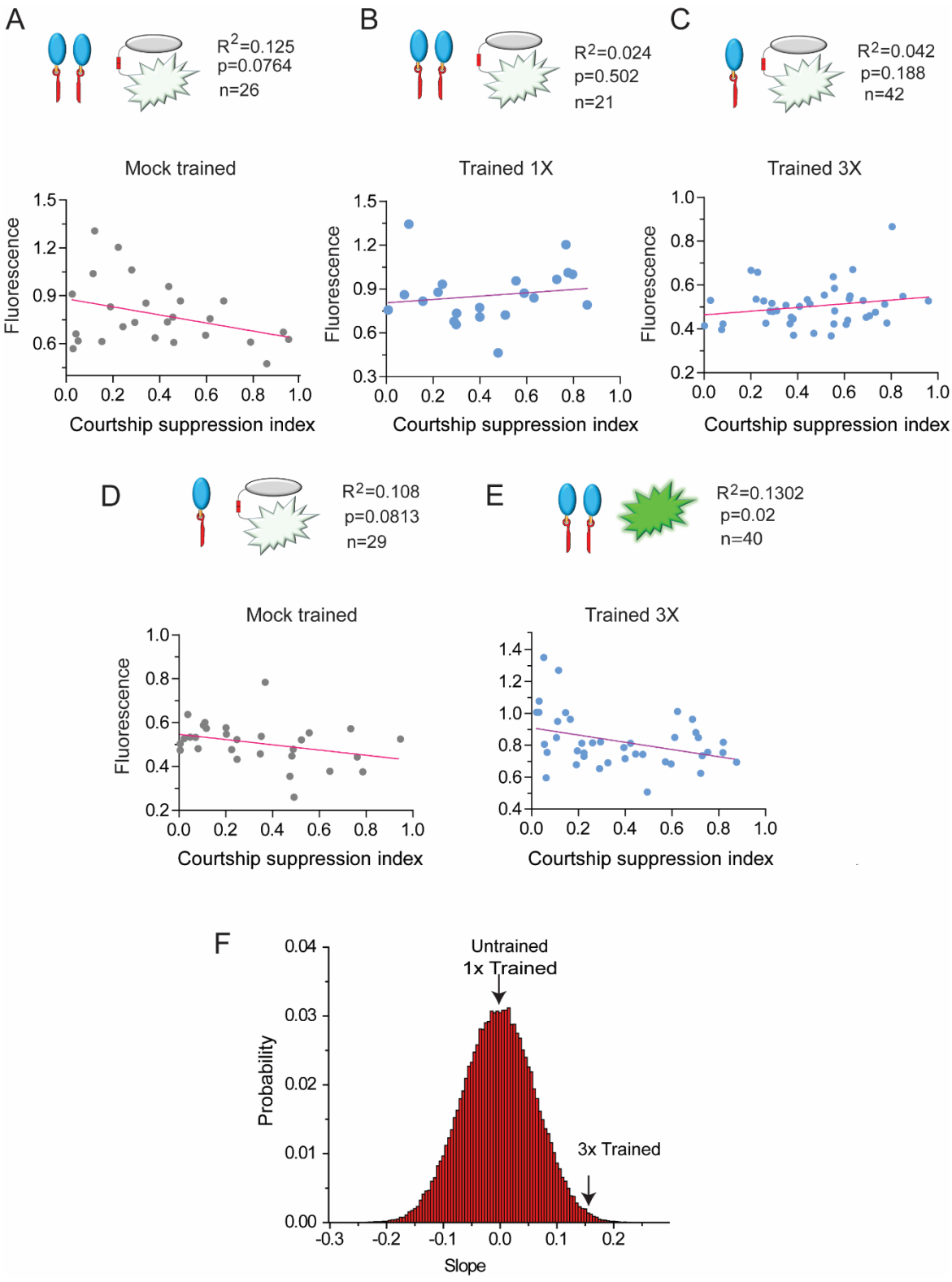

Figure 6.4 Control groups of Figure 6.3 do not show positive correlation between memory strength and Orb2-aggregation.

Control groups do no show correlation between courtship suppression and GFP intensity in Ɣ lobe:

A) Mock trained flies (ElavGal4::UAS-GFPdark; Orb2-TEVN/Orb2-TEVC)
B) 1X trained flies (ElavGal4::UAS-GFPdark; Orb2-TEVN/Orb2-TEVC)
C) 3X trained flies with one half of TEV protease (ElavGal4::UAS-GFPdark; Orb2-TEVN/Orb2)
D) Mock trained flies with one half of TEV protease (ElavGal4::UAS-GFPdark; Orb2-TEVN/Orb2)
E) GFP reporter that does not depend on TEV protease activity (ElavGal4::UAS-GFP; Orb2-TEVN/Orb2-TEVC)
F) Monte Carlo simulation of the memory index and fluorescence intensity of the data from figure 6.3&6.4.

Linear regression was used to analysis the correlation between courtship suppression index and GFP intensity. Only the positive courtship suppression index was plotted.

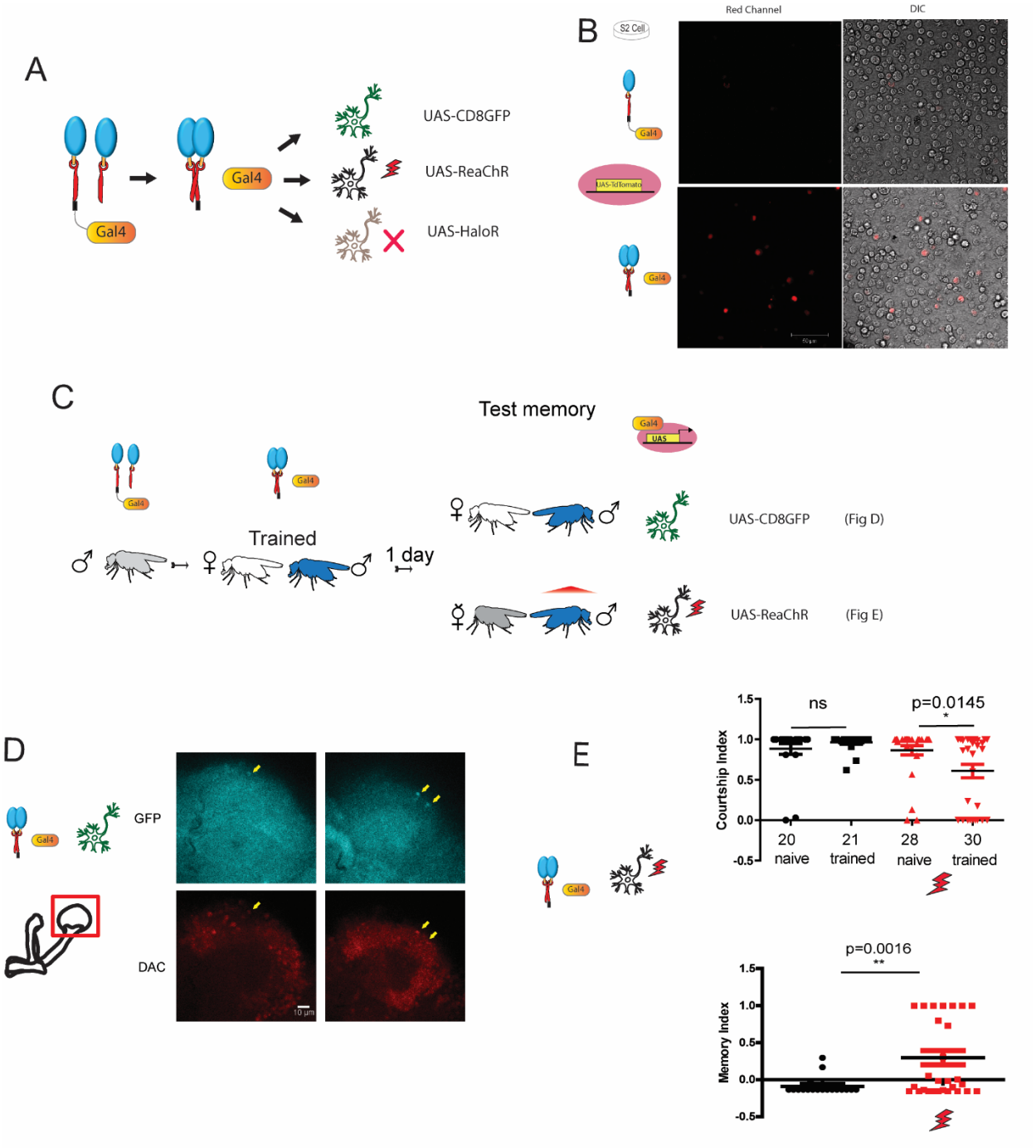

Figure 6.5 Artificially activating neurons in which Orb2 oligomerizes can retrieve a memory.

A) Schematics of coupling of Orb2-dependent TEV protease reconstitution to transcription activation. The Gal4 transcription factor is fused to Orb2-TEVN with a linker carrying TevS. Oligomerization of Orb2 reconstitutes TEV protease activity then cleaves Gal4 off the

oligomers. Gal4 can mediate transcription activation of various UAS-reporter, such as GFP, red-shifted channelrhodopsin and holorhodopsin.

B) In S2 cell, reconstituted TEV protease by Orb2 can activate expression of UAS-TdTomato. While expression of only the Orb2-TEVN-Gal4 does not lead to transcription activity. Scale bar: 50um.

C) Schematics of visualizing and activating the neurons in which Orb2 oligomerizes. Male flies were trained in courtship suppression memory paradigm by prolonged exposure to mated unreceptive female. Orb2 oligomerizes during training and consolidation, freeing Gal4 to the nucleus and transcribing target genes. Expression of GFP allows visualization of the neurons. Expression of the red-shifted channelrhodopsin (UAS-ReaChR) allows stimulation of the neurons during testing. To test whether a memory can be artificially recalled by stimulating the labelled neurons, the males are tested against decapitated virgin female, which should not elicit courtship suppression memory unless the memory circuit is stimulated.

D) Sparse labeling of neurons in the mushroom body calyx can be detected after training. Images show one side of mushroom body cell body region (calyx) of the trained flies. Yellow arrows indicate positive labelling. DAC: dachshund, a mushroom body specific transcription factor. Scale bar: 10um.

E) Activating neurons labelled by Orb2 oligomerization is sufficient to retrieve a memory. Flies are tested with decapitated virgin female. Upper panel: courtship index of naïve (untrained) and trained flies in both unstimulated (black) and light-activated (red) groups. The lower the courtship, the better the memory. Bottom panel: memory index calculated for both unstimulated (black) and light-activated (red) groups. Unpaired t-test is used. *$p < 0.05$, **$p < 0.01$.

Chapter 7. Discussion and Future Direction

A memory, in addition to being stored, must be accessed, retrieved and able to elicit the proper behavioral response and perturbation of any of these steps can interfere with behavioral display of memory. While previous studies have found that the prion-like domain (Keleman et al., 2007; Krüttner et al., 2012) and aggregation of Orb2 (Majumdar et al., 2012) is required for memory, it is unclear when and how long Orb2 activity is required and whether Orb2 aggregation is one of the limiting steps in memory formation. Here, by acutely and reversibly inactivating Orb2 at different stages of memory only in the adult neurons, by artificially facilitating Orb2 aggregation, and by visualizing aggregated Orb2 following behavioral training, I find that Orb2 is required for the formation, persistent storage, and retrieval/expression of memory; that facilitation of Orb2 aggregation facilitates long-lasting memory formation; and extent of Orb2 aggregation in the γ-lobe neurons is predictive of memory strength. As I discuss below, taken together, the studies in *Aplysia* (Si et al., 2010), fly (Krüttner et al., 2012; Kruttner et al., 2015; Majumdar et al., 2012) and in mouse (Fioriti et al., 2015; Stephan et al., 2015) could be best explained if one considers that self-sustaining aggregates of CPEB is a constituents of the memory engram.

7.1 A system to inactivate Orb2 protein

In my thesis, I utilized the TEV protease cleavage system to inactivate Orb2 directly on the protein level. This methodology has the advantage over the conventional gene knock-out and RNAi knock-down in the following aspects: first, the inactivation is fast and does not depend on the decay of existing proteins; second, it can be designed to target various conformations of protein; and third, it is reversible. The efficiency of TEV protease cleavage depends on the target, the surrounding protein complex, expression and stability of the protease, localization of the target

and protease, and cell types. In the *Drosophila* S2 cell line, the cleavage efficiency is >80% for all the targets tested, however, the efficiency of cleaving Orb2 in the fly head neurons is ~50% on average. This could be due to the fact that the TevS recognition site is not fully exposed *in vivo*, possibly due to shielding by other protein complexes, or the induction of TEV protease in the fly head may not be as much as, or as stable as those expressed in S2 cell, or the highly polarized neurons may not have uniformed distribution of the protease, or Orb2 is relatively concentrated at some spots where TEV protease cannot reach. Despite ~50% cleavage efficiency, there was a significant effect on long-term memory, suggesting 50% of Orb2 protein in the whole brain is insufficient for either encoding or retrieval of long-term memory. I do not know whether all of the Orb2 expressing neurons lost 50% of the protein or in a subset of critical neurons 100% of the protein is lost.

Inactivation of Orb2 protein in the neurons disrupts long-term memory. The exact mechanism *in vivo* is unclear however, evidence from *in vitro* translation assay suggests inactivation of Orb2 results in disregulation of Orb2 target mRNA translation. Future experiments to address the biochemical outcome of inactivation of Orb2 *in vivo* is to genetically introduce an Orb2-dependent translational reporter and assay Orb2-dependent translation spatially and temporally. This will also allow us to address the mechanism of the memory recovery, at least whether it is dependent on Orb2 translation activity. Also it's unclear at this stage in which subcellular compartments Orb2 is critical for memory: cell body, presynaptic or post-synaptic compartment? And how memory encoding and retrieval are affected if inactivating Orb2 in different compartments? Tools that restrict the TEV protease to different compartments are necessary to address these questions.

7.2 Aggregated CPEB/Orb2 as a putative memory engram.

There are three ways to study memory engram: observing the engram, erasing the engram, and artificially engaging the engram. I have interrogated aggregated CPEB/Orb2 as a memory engram in all three aspects:

First, by coupling Orb2 aggregation to TEV protease activity, I was able to observe the relationship between aggregation of Orb2 and memory strength. This is to my knowledge one of the very few molecular events that correlates with memory strength. One drawback of the splitTEV reconstitution system is that it's actually scoring for dimerization and does not differentiate whether enhanced TEV protease activity is due to one large Orb2 aggregate or a number of small aggregates (including Orb2 dimers). If Orb2 dimers are the only source for reconstituted TEV activity, the following scenario should be considered: Orb2-TEVN and Orb2-TEVC can form homo- or hetero-dimer whereas only hetero-dimer can confer the protease activity. If this is the case, only 1/3 of the Orb2 dimers are scored. If Orb2 forms trimer and as long as one Orb2-TEVN and one Orb2-TEVC are in the same trimer, the TEV protease can be reconstituted. In this case reconstituted TEV protease activity is representing 3/4 of Orb2 trimers. If considering more Orb2 molecules in the same aggregates, the protease activity is representing higher portion of Orb2 x-mers. If considering full length TEV protease has the maximum efficiency, the fact that in S2 cells and in mushroom body γ neurons reconstituted TEV protease by Orb2 have similar enzymic activity compared to full length TEV, suggested Orb2 aggregates should be more than dimers.

Second, destabilizing Orb2 aggregates by either interfering with the endogenous protein phosphorylation pathway (TobRNAi) or exogenously expressing an anti-amyloidogenic peptide (QBP1) affect specifically the long-term memory. These results are consistent with the idea that oligomerization of Orb2 is a putative substrate for memory and disruption of it results in impairment of memory. However, since RNAi has a slow kinetics and is irreversible, and QBP1

can only affect de novo orb2 oligomerization, these reagents do not allow temporal interference. Therefore, I designed the transient TEV protease cleavage system, which inactivate both monomeric and oligomeric Orb2 protein and test the temporal requirement of Orb2. Inactivation of Orb2 protein during encoding of memory interferes with long-term memory consolidation that cannot be rescued when Orb2 is re-expressed beyond consolidation period (>6h after training). Inactivation of Orb2 protein during retrieval temporally blocks the access to the stored memory while memory can be recovered once >80% of the protein level is re-expressed. These results suggest Orb2 is a biochemical substrate for both encoding and retrieval of long-term memory.

Third, artificially facilitating Orb2 aggregation by a yeast chaperone JJJ2 lowers the threshold for long-term memory consolidation. This satisfied the sufficiency of oligomeric Orb2 in forming long-term memory. Here we only used JJJ2 as a tool to enhance Orb2 aggregation. The exact mechanism of how JJJ2 facilitates Orb2 aggregation is waiting to be addressed. We also don't have temporal control over JJJ2 expression because the leaky expression from the UAS-JJJ2 transgene alone is sufficient to generate memory enhancement. Indeed, memory improvement should be a rare phenomenon because to improve memory by mere activation of a molecular process is not enough - it must be engaged in the right cell (and synapse), at the right amount and requires accompanying changes in other molecular processes. This would suggest JJJ2 is correctly engaged in the right place during either memory encoding or retrieval. Other tools that can allow temporally and spatially engaging Orb2 oligomerization such as light-inducible oligomerization is the future direction to address where and when Orb2 aggregation is sufficient to encode or retrieve a memory.

7.3 Memory Recovery

Intriguingly I find that, once established, specific memory can recover in an Orb2-dependent manner. While the exact mechanism of memory recovery upon restoration of Orb2 is unclear, based on the fact that inactivation by TEV protease leaves ~50% of the protein in the neurons, I envision the following possibilities. Small amounts of full length Orb2 aggregates or aggregates of just the N-terminal domain could induce aggregation of monomeric Orb2 (Hervas et al., 2016; Khan et al., 2015; Krüttner et al., 2012). Therefore, one possibility is that the residual uncleaved aggregated Orb2 or the cleaved aggregated N-terminal domain, although inadequate for full translational activation and expression of memory, reconstitutes the memory trace once monomeric Orb2 protein level is restored. A formal test of this possibility would be if Orb2 aggregates are completely eliminated after memory formation, memory should never recover. Although technically it has not been feasible to accomplish this in *Drosophila*, recent studies in mice is consistent with this possibility: removal of CPEB3 from the genomic locus results in loss of established memory that cannot be rescued by restoring CPEB3 expression after few weeks (Fioriti et al., 2015). The other possibilities are that Orb2 has distinct and independent function in memory storage and memory retrieval. Alternatively, there's some process upstream of Orb2 that regulates Orb2 aggregation-once Orb2 protein level is restored the aggregation and translation is restored by this upstream process to the level adequate for memory. In all these scenarios continued Orb2-dependent translation is required for manifestation of memory days after formation.

7.4 Memory enhancement

We find that JJJ2 can facilitate, but can't initiate long-term memory formation suggesting there are restrictions to long-term memory formation and likely Orb2 aggregation that JJJ2 by itself can't overcome. Availability of monomeric Orb2A protein, a substrate of JJJ2, could be one of the restricting components, since it is extremely low abundant (Krüttner et al., 2012; Majumdar

et al., 2012) and has a very short half-life (White-Grindley et al., 2014a). What JJJ2 most likely does is lower the threshold of Orb2A protein requireed to initiate Orb2 aggregation.

Among chaperones, the Hsp40 family of chaperones is most expanded (Kampinga and Craig, 2010). JJJ2, a low abundant nonessential gene is reported (SGD database) to interact physically only with 4 proteins in yeast suggesting JJJ2 is likely to have a limited set of targets. Based on the findings that JJJ2 enhances Orb2 aggregation, Orb2-dependent translation and Orb2-dependent memory, we postulate that JJJ2-Orb2 interaction is important for memory and Orb2-aggregation is at least a rate-limiting step in gating long-lasting memory formation. However, this does not mean that the memory enhancing effect of JJJ2 is mediated exclusively through Orb2. Also JJJ2 serves as a tool but not necessarily provides mechanistic insights into how chaperones control Orb2 aggregation in adult fly brain. However, it raises the possibility that functional protein aggregation in the brain can be guided by molecular chaperones and there may be a functional equivalent of JJJ2 in *Drosophila* and other species. Future studies will focus on what controls the availability of Orb2A protein, how JJJ2 induces Orb2A conformational switch, in which cell types and when JJJ2 is important for memory and what is the *Drosophila* equivalent of JJJ2.

7.5 The synaptic tagging and capture theory

How can a self-sustaining state of a protein synthesis regulator explain persistence, enhancement and under certain circumstances recovery of a memory? Perhaps these observations can be best explained in the framework of synaptic "tagging". According to synaptic "tagging" a synapse undergoing a long-term change creates a molecular state that "tags" the activated synapse. The tag allows the plasticity-related molecules to be captured/utilized/synthesized in the activated synapse (Frey and Morris, 1997, 1998; Martin et al., 1997; Redondo and Morris, 2011). The self-sustaining aggregated CPEB has been postulated by us and others as a component of synaptic tag.

Once created, the aggregated CPEB allows the synapse to maintain its altered state by continuously capturing and translating specific mRNAs only at the activated synapse (Si et al., 2010) (Khan et al., 2015; Mastushita-Sakai et al., 2010). My thesis work extends these cellular functions of Orb2/CPEB aggregates to the behavior level:

1) Persistence: behavioral memory requires Orb2-dependent protein synthesis days after formation because the continued presence of Orb2 as a tag is required to maintain and express memory. 2) Recovery: when Orb2 is inactivated during acquisition and consolidation, memory can't be recovered with newly synthesized Orb2 because the tag was never formed- however, once formed, owing to its self-sustaining properties, even a small amount of Orb2 can recreate the tag allowing recovery of memory. 3) Enhancement: facilitation of Orb2 aggregation by JJJ2 facilitates formation of the synaptic tag. This allows suboptimal training induced plasticity related mRNAs that otherwise would not be utilized by the synapse now to be captured and utilized by the synapse to produce long-term memory. This is akin to expression of activated CREB that produces cell wide facilitation that can be captured by an activated synapse by subthreshold stimuli to produce long-term change (Han et al., 2007; Rogerson et al., 2014; Sano et al., 2014; Sargin et al., 2013; Suzuki et al., 2011; Viosca et al., 2009; Yiu et al., 2014; Zhou et al., 2009). Intriguingly we find that more of JJJ2 is not necessarily conducive to better memory, consistent with other reports that low but not high levels of chaperons facilitate prion-like aggregation (Higurashi et al., 2008). Integration of JJJ2 at attP40 site serendipitously provided the appropriate amount to aid memory formation.

Future direction to test the Orb2 as a synaptic tag is to visualize its recruitment to activated synapses once animals formed long-term memory, and ask whether this tag can capture new Orb2 protein as well as target mRNA for translation.

7.6 Visualization of molecular signature of a long-lasting memory

An independent measure of memory in addition to behavioral readout is necessary to interrogate the mechanism of persistence as well as decay of memory. Previously memory traces have been visualized at the neuronal activity level using immediate early genes (Liu et al., 2012; Ramirez et al., 2013; Redondo et al., 2014; Reijmers et al., 2007; Ryan et al., 2015; Tanaka et al., 2014; Tayler et al., 2013) or calcium sensors (Akalal et al., 2010; Cervantes-Sandoval and Davis, 2012; Yu et al., 2006; Yu et al., 2005). Likewise autocatalytic calcium calmodulin-dependent kinase II (CamKII) (Sanhueza and Lisman, 2013), a specific isoform of atypical protein kinase C, PKMξ (Sacktor, 2011), ratio of phosphorylated-CREB to total CREB or the level of postsynaptic GluR2 (Migues et al., 2010), and DNA modification (Zovkic et al., 2013) have been reported as a biochemical substrates of long-term memory. However, some of these molecules are involved in a broad range of physiological functions, rendering them difficult to always associate with memory processes per se. The specific involvement of CPEB/Orb2 aggregates perhaps provides a more selective tool for "visualization" of memory, at least in *Drosophila*.

The *Drosophila* mushroom body is generally believed to be important for long-lasting memory (Guven-Ozkan and Davis, 2014; Heisenberg, 2003). Approximately 2000 neurons in the mushroom body are divided into three distinct lobes (α/β, α'/β' and γ) and seven distinct cell types (Aso et al., 2009; Aso et al., 2014). Multiple studies suggest the vertical branch of α/β lobe is required for memory persisting for 9-24h (Krashes et al., 2007; Pascual and Preat, 2001; Yu et al., 2006), while the γ lobe is important for memory beyond a day (18-48h) (Akalal et al., 2010; Keleman et al., 2007). I find aggregated-Orb2 in the γ lobe is positively correlated with memory strength. It is unclear how many neurons or whether a specific set of neurons in the γ lobe are recruited for a given memory. Likewise, although Orb2-aggregation in the γ lobe is predictive of

long-term memory, Orb2 does not necessarily only aggregate in the γ lobe. Nonetheless it allows molecular visualization of memory in the engram cells and offers a possibility to interrogate the molecular basis of memory loss.

7.7 Conclusion

My thesis studies have tried to address the spatial and temporal requirement of Orb2 during different phases of memory. I discovered that Orb2 activity is required for both encoding and retrieval of long-term memory; interference with Orb2 aggregation blocks while facilitation of Orb2 aggregation enhances memory; and oligomerization of Orb2 in specific neurons can be a readout of memory strength. These are the essence of a memory engram, or a physical substrate of memory. The unique biophysical property of Orb2 -- a self-perpetuating prion-like aggregate – is most likely the core mediating these aspects. My studies substantially strengthen the functional amyloid hypothesis in memory.

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