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DISSERTATION

Titel der Dissertation

The role of the *Drosophila* CPEB protein Orb2 in long-term memory.

Verfasser

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Synopsis

Long-term memory formation is thought to reflect experience dependent modifications at specific synapses. These are, at least in part, mediated by local translation of mRNAs to strengthen existing synapses and to build up new connections. Because mRNAs are in theory available to all synapses of a neuron the existence of a synaptic tag has been postulated. Such a synaptic tag could either allow translation of dormant mRNAs only in specific synapses or capture the proteins required for the modifications of synaptic connections.

CPEB proteins are potential candidates for such a synaptic tag for several reasons— perhaps most importantly this family of proteins is able to control mRNA translation.

Here we demonstrate that Orb2, the *Drosophila* CPEB homologue, is required during courtship long-term memory but dispensable for short-term memory. In a series of experiments we mapped the requirements of Orb2 to the mushroom body, a well-known learning center of the *Drosophila* brain. While Orb2 is required developmentally for other processes, within the mushroom body it is acutely required during learning.

To address the molecular mechanism behind Orb2 function we generated a novel Orb2 allele, which replaces most of the Orb2 reading frame with an acceptor site for site-specific recombination. We found that both isoforms, Orb2A and Orb2B are critically required for long-term memory formation. Genetic complementation experiments suggested that Orb2 might form complexes. A series of biochemical experiments also suggest the presence of higher order oligomers. Preliminary experiments demonstrate that at least some of these oligomers are dependent on a glutamine stretch at the N-terminus of Orb2. This domain has previously been suggested to confer prionic properties to the protein and when removed flies are no longer able to form functional long-term memory.

Synopse

Langzeitgedächtnis spiegelt Veränderungen von Synapsen wieder. Diese Veränderungen, wie z.B eine Verstärkung oder Neubildung der Synapse, werden zum Teil durch lokale Translation von mRNA ermöglicht. Da diese mRNA aber im Prinzip allen Synapsen zugänglich ist wurde die Hypothese des so genannten “synaptic tagging” entwickelt. Diese postuliert eine Markierung der Synapsen, welche entweder die lokale Translation ausschließlich in den spezifisch markierten Synapsen ermöglicht, oder die benötigten Proteine in die markierten Synapsen akquiriert.

CPEB Proteine erscheinen als idealer Kandidat für eine solche synaptische Markierung, da sie in der Lage sind die Translation von mRNA zu kontrollieren.

Hier demonstrieren wir, dass Orb2, das homologe CPEB Protein in Fruchtfiegen, für das Erinnerungsvermögen, in einem für das Paarungsverhalten spezifischen Versuchsaufbau, benötigt wird. In einer Reihe von Experimenten gelang es uns zu zeigen, dass Orb2 im Mushroombody von Fruchtfiegen, einer Gehirnstruktur die schon länger dafür bekannt war für Gedächtnis von Bedeutung zu sein, benötigt wird. Dabei ist Orb2 nur unmittelbar während der Zeit des Trainings von Bedeutung, nicht aber für das Wiederaufrufen des Gedächtnisses. Auch ist das Protein nur für Langzeit- aber nicht Kurzzeitgedächtnis von Bedeutung.

Um die molekularen Mechanismen dieser Erkenntnisse zu studieren, generierten wir ein weiteres Allel, welches die Orb2 spezifische Gensequenz durch eine Integrationsequenz ersetzt. Diese Integrationsequenz ermöglichte es uns das endogene Orb2 Protein durch modifizierte Versionen zu ersetzen. Wir zeigen, dass beide Isoformen, Orb2A und Orb2B, für die Bildung des Langzeitgedächtnisses von zentraler Bedeutung sind. Außerdem konnten wir genetisch und biochemisch nachweisen, dass Orb2 als Oligomer auftritt und in dieser Form funktionell aktiv ist.

1. Introduction

Plasticity of behaviors has evolved to allow organisms to cope with changing external environments and changing internal states. Memories allow the modifications of behaviors to be stored over time, to be combined, and finally to be recalled (Kandel, 1976; Milner et al., 1998). Although the formation and storage of memory is very cost intensive (Burns et al., 2010; Dukas, 1999), it gives an enormous advantage in the battlefield of life, where only the fittest, and therefore best adapted, will survive. It is thus not very surprising that almost all animal behaviors can be modified by experience– from the simple gill withdrawal reflex of the sea slug, *Aplysia californica* (Kupfermann and Carew, 1974), to the proboscis extension reflex in the honeybee (Braun and Bicker, 1992), to the courtship behavior of the fruit fly, *Drosophila melanogaster* (reviewed in Dickson, 2008), up to classical conditioning in mammals first described by Pavlov.

For more than a century, scientists have been interested in how nervous systems can sense and integrate external and internal stimuli to control the animal's behavior accordingly (Kandel, 1976; Milner et al., 1998). The enormous cellular complexity of nervous systems, as first illustrated by Santiago Ramon y Cajal (Ramon, 1952), makes it a hard task to answer these questions. Partly as a result of this complexity, many questions remain unanswered.

Because of the enormous cellular complexity, Erik Kandel and Seymour Benzer introduced the so-called reductionists approach to the field of behavior thirty years ago. They proposed to use simple organisms (like *Aplysia californica* and *Drosophila melanogaster*), with smaller nervous systems and simple but robust behaviors, for studies that aimed at identifying molecules, mechanisms and neuronal networks underlying behavior (Benzer, 1971; Hotta and Benzer, 1973; Kandel, 1979).

In contrast to the mammalian brain that consist of billions of neurons, *Aplysia* has only 20.000 neurons (Kandel, 1979). Nevertheless, *Aplysia* exhibits behaviors that can be modified by experience (reviewed in Castellucci, 2008).

When a weak tactile stimulus is applied to the *Aplysia*'s soft body its siphon and gill are withdrawn into the mantle cavity for protection. This reflex can be modified by different forms of learning: habituation (Kupfermann et al., 1970), sensitization and classical conditioning (Bao et al., 1998; Hawkins et al., 1998). The different forms of learning can be distinguished by their strength, duration, and the stimuli used for training (Rosenzweig et al., 1993). In general, weaker experiences produce short-lived memories whereas stronger experiences produce long-lived memories (Castellucci et al., 1986; Pinsker et al., 1973).

But what are the cellular differences between these different forms of learning? How can the nervous system make these changes long lasting? How can short-term and long-term memories be differentiated within a nervous system? And why should we use fruit flies as a model organism to study memory and learning?

Here I aim to give an overview of the different forms of learning and memories and the known mechanisms and key players behind them. I will draw the main focus of these findings to *Drosophila* for reasons that I hope will become apparent in the subsequent sections.

1.1.1 *Drosophila* as a model organism for memory and learning

During the past decades, *Drosophila* has become one of the key organisms in neurobiology (reviewed in Castellucci, 2008). The success of *Drosophila* in neurobiology derives from several factors. First, fruit flies have relatively few neurons compared to mammals (Castellucci, 2008). This attribute makes it easier to trace the relevant circuits required for a given behavior. Second, *Drosophila* shows a variety of well-characterized robust innate behaviours that can, importantly, be modified by experience (Benzer, 1971; Heisenberg, 1997; Hotta and Benzer, 1973). Examples are: courtship behavior, circadian rhythms, CO₂ avoidance and aggression. Third, *Drosophila* has numerous genetic tools available. The tools range from the fast generation time, which has allowed forward genetic screens, to the current range of reverse genetic tools that allow

the ready manipulation of both genes and neuronal function. Of particular importance has been the development of the Gal4-UAS expression system. The GAL4-UAS system uses a bipartite system where the yeast transcription factor GAL4 can be used combinatorially to drive any gene of interest under the upstream activating sequence (UAS) (Brand and Perrimon, 1993). Multiple generations of the GAL4-UAS system now allow expression of virtually any protein in a tissue and time specific manner (Pfeiffer et al., 2008; Pfeiffer et al., 2011).

But what can *Drosophila* learn and remember? Here I will give a brief summary of some of the more well known examples and will hopefully convince you that the answer to this question is 'a lot'.

One of the first, and still one of the most widely used assays, is an olfactory memory assay, the so-called T-maze. In this assay, flies learn to associate an odour (conditioned stimulus - CS) with an electric shock (unconditioned stimulus - US). Using a T-maze, flies can choose between the conditioned and the unconditioned odour (Quinn et al., 1974; Tully and Quinn, 1985a). This assay is the *Drosophila* version of aversive conditioning. In the *Drosophila* field, this assay has led to most of our knowledge on learning and memory.

The conditioning response can also be elicited by rewards. Specifically, the flies can learn to associate odours with food reward (Krashes and Waddell, 2008; Tempel et al., 1983). In the proboscis extension reflex assay insects are trained to suppress their reflex towards a gustatory cue, which can be either aversive or attractive (Chabaud et al., 2006; DeJeanne et al., 1985; Medioni and Vaysse, 1975).

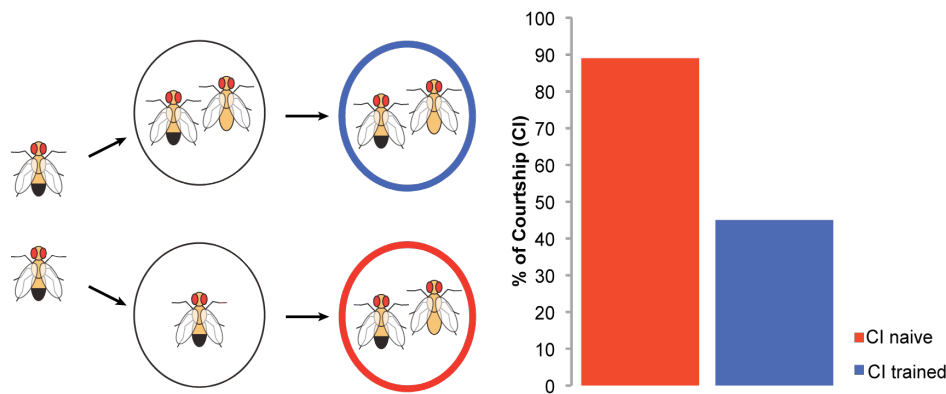
Flies can differentiate between different shapes and colors while navigating through a LED arena either during flight (Brembs and Heisenberg, 2000; Dill et al., 1993; Guo and Gotz, 1997; Xia et al., 1997) or while walking (Strauss and Pichler, 1998). Importantly, it has recently been shown that they can have spatial memory based on these cues (Ofstad et al., 2011), similarly to higher organism (Derdikman and Moser, 2011).

Finally, the so-called ‘courtship conditioning’ assay has been used to study learning and memory. This assay exploits male flies ability to learn to distinguish between sexually receptive and unreceptive females. This type of learning is behaviorally highly relevant for the male fly, which makes it an attractive paradigm to study memory in *Drosophila* (McBride *et al.*, 1999).

1.1.2 The courtship conditioning paradigm

For *Drosophila*, as for most other organisms, successful reproduction is a major goal. Successful reproduction not only entails offspring production, but also producing offspring with the highest possible fitness. It is thus of importance for both sexes to choose the best possible mating option available. Courtship behavior is a robust innate behavior (Baker *et al.*, 2001; Hall, 1994) and the courtship rituals allow both sexes to assess the quality of their potential partner prior to copulation (Hall, 1994; Spieth, 1974; Sturtevant, 1915). Amongst the cues used in making the assessment, the auditory system is employed during the courtship song (Markow, 1987; Rybak *et al.*, 2002), the olfactory system during pheromones detection (Billeter *et al.*, 2009; Kurtovic *et al.*, 2007; Markow, 1987; Rybak *et al.*, 2002) and the visual system during size determination (reviewed in Dickson, 2008). It is important to highlight at this point that the majority of these behaviors are hard-wired into the nervous system– in other words, they are not learnt behaviors. Nonetheless, numerous aspects of courtship can be modified by experience. These modifications can be of two varieties: 1) directly modulated by molecular cues or 2) learnt. As an example of modulation of behavior by direct molecular cues, females become unreceptive to copulation attempts for about one week after mating (Manning, 1962). During this time the mated female shows increased egg laying rates (Bloch Qazi *et al.*, 2003; Kubli, 2003) and actively rejects other males (Connolly and Cook, 1973). This behavior is not learnt, but rather directly modified by a small peptide, the sex peptide, delivered with male seminal fluid (Burnet *et al.*, 1973). However, females can learn. A nice example of this comes from the observation that females can determine the local

pool of males and make mating choices based on learning. Specifically, when raised with only small males they will change their preference for male size when later offered the option of small or large partners (Dukas, 2005b). Likewise, male flies can also learn. Sexually naïve *Drosophila* males initially court all sorts of females: immature virgins, receptive virgins, mated females, con-specific fruit flies, and even decapitated females. However, over time they learn to selectively avoid courtship towards immature virgins (Ackerman and Siegel, 1986; Gailey et al., 1984; Tompkins et al., 1983), mated females (McBride et al., 1999), con-specific females (Dukas, 2004), and decapitated females (Ejima et al., 2005; Spieth, 1966), while improving their courtship towards receptive mature virgins (Dukas, 2005a). A courtship assay has been developed to quantitatively test the amount and duration of learning (Fig.1). In this assay, males that had already experienced mated females during the training period exhibit drastically reduced courtship during the test towards mated females, but exhibit normal courtship toward mature virgins (McBride et al., 1999). This indicates that these males learned to differentiate receptive from unreceptive females. Depending on the training protocol and length of training flies have memories that can last from minutes to even days (McBride et al., 1999).



$$\text{Learning Index (LI)} = \frac{\overline{CI}_{\text{naive}} - \overline{CI}_{\text{trained}}}{\overline{CI}_{\text{naive}}} \times 100$$

Figure 1 The Courtship Conditioning paradigm

During a training period (1hr for STM, 5h for LTM) naïve males get paired with a previously mated female. Control males undergo sham training without female. In a subsequent test (1h after training for STM, 24h after training for LTM) both are videotaped for 10min with mated females. While trained wild type males selectively learned to suppress their courtship towards mated females, naïve control males still vigorously court them. From the resulting video the Courtship Index (CI), which is the time the male spends in courtship is calculated. The resulting CIs are used to calculate the Learning Index (LI).

The courtship assay has been used to test the molecular and cellular underpinnings of learning and memory. At a molecular level, the pheromone, cis-vaccenyl-acetate (cVA), and its receptor, Or67d, are key players in courtship. cVA may be one of the major chemical cues that both female and male flies use to communicate mating status (Kurtovic et al., 2007). A cellular focus of learning is still largely unknown; however, in the odor-shock paradigm dopaminergic neurons have recently been shown to be important elements of the circuits that allow *Drosophila* to learn (Krashes et al., 2009). cVA and other cues are integrated within the fly brain with a likely important role for dopaminergic neurons. At a molecular level numerous experiments have identified elements of the molecular pathways that probably operate within these central-integrating cells. These molecules will be highlighted in the next section on ‘memory phases’

Starting from the external cues, one major player is cVA. cVA gets transferred to the female together with sperm and peptides during copulation (Butterworth, 1969; Ejima et al., 2007). Interestingly, it has disparate effects in the sexes: while stimulating receptivity in females (Kurtovic et al., 2007), it reduces courtship in males (Ejima et al., 2007; Kurtovic et al., 2007). The ability of cVA to mediate various behavioural responses together with its presence in mated females, but absence in mature virgins, makes this pheromone a putative substrate to discriminate between mated and receptive females. In order to learn to discriminate between these two types of females, males could use cVA either as an unconditioned stimulus (US) or as a conditioned stimulus (CS). If cVA would act as US then the male should associate the presence of cVA with other female pheromones. In case cVA is acting as CS rejection by the female or unsuccessful courtship could serve as US to be associated with cVA. Surprisingly, recent data (Keleman et al., unpublished) suggests that cVA does not function as a classical CS but nevertheless the behavioural response to cVA is modulated by rejection or unsuccessful courtship. Specifically, cVA is not required during the training period but rather is only required during a subsequent testing period (Keleman et al., unpublished). This implies that cVA is not the US but rather rejection sensitizes the males perception to cVA. This sensitization leads to an increased ability of the males to discriminate mated from unmated females, based on the presence of cVA, after training.

As we move from the external cues to the internal processing, dopaminergic neurons are key candidates to relay the reinforcement signals required for learning (Krashes et al., 2009; Schwaerzel et al., 2003; Selcho et al., 2009; Tempel et al., 1984; Zhang et al., 2008). A likely site for experience-dependent modulation of pheromone responses by dopamine is the mushroom body (MB), a well known center for olfactory memory in insects (Heisenberg, 2003). Most of this structure consists of the Kenyon cells (about 2,500 in *Drosophila*). The Kenyon cell bodies are densely packed above and beside the calyces, their dendritic field lie in the dorsocaudal cell body rind, and their axons form the peduncle and the lobes (Heisenberg, 2003) (Fig.2). The MB lobes express the

essential courtship gene *fruitless* (*fru*) (Manoli et al., 2005). Disrupting *fru* in these neurons blocks experience dependent courtship modulation (Manoli et al., 2005). An essential gene for long-term memory, *orb2*, is critically required in MB- γ -lobes, making them a likely site of learning (Keleman et al., 2007).

In summary, cVA plays a key role as a pheromone that allows discrimination of mated and virgin females. This learning likely involves the reinforcement signal conveyed via dopaminergic neurons to the major learning center in insects, the mushroom body.

In the next section I will describe the forms of memory that exist in *Drosophila* and some of their known molecular correlates.

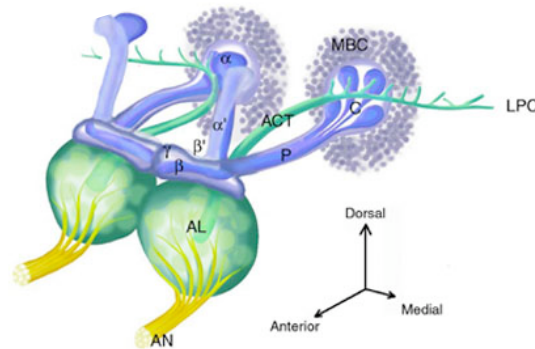


Figure 2 Organization of the *Drosophila* Mushroom body

Odor information is conveyed from about 1500 olfactory sensory neurons on each antenna through the antennal nerves (AN) to 43 glomeruli in each antennal lobe (AL), where these axons synapse to the antennal lobe relay neurons. Relay neurons with cell bodies in the antennal lobe transmit olfactory information through the antennal cerebral tract (ACT) to synapse on the dendrites of mushroom body Kenyon cells (MBC). The MBC dendrites are located in a neuropil structure known as the calyx (C). The MB axons are bundled to form the peduncle (P), which projects toward the anterior face of the brain to synapse on the dendrites of follower neurons in neuropil regions termed the lobes. Some lobes are oriented vertically and some horizontally with respect to the fly head. The α/β MB neurons project axon branches into the vertically oriented α lobe and the horizontally oriented β lobe (blue structures). The α'/β' neurons project axon branches into similarly oriented lobes termed α' and β' (gray structures). The γ MB neurons do not branch and project only into the horizontally oriented γ lobe (purple-blue structures). [Figure adapted from (McGuire et al., 2001)]

1.1.3 Different memory phases in *Drosophila*

Memory can be divided into multiple types based on the manipulation required to achieve them as well as their subsequent durations. Interestingly, the different types of memory have distinct neuronal and genetic requirements, which has been used to argue against one neuronal flow in memory consolidation from short- to long-term memory (Ng et al., 1991; Rosenzweig et al., 1993). Based on all these criteria, the memory types were classified as short-term memory (STM), mid-term memory (MTM) and long-term memory (LTM) (Becker et al., 1994; Ng et al., 1991; Rosenzweig et al., 1993). Short- and mid-term memories use covalent modification of existing proteins, whereas long-term memory requires synthesis of new proteins (Castellucci et al., 1989; Flexner et al., 1963; Kandel, 1989, 2001; Montarolo et al., 1986).

The study of mutant and transgenic flies has genetically dissected memory formation further into four distinct phases: short-term memory, mid-term memory, anesthesia-resistant memory (ARM) and long-term memory (Isabel et al., 2004). Mutants such as *dunce* (*dnc*), *rutabaga* (*rut*), *amnesiac* (*amn*) and *radish* (*rsh*) were identified in the classic genetic screens for mutants defective in the before mentioned T-maze olfactory conditioning assay (Dudai et al., 1976; Folkers et al., 1993; Livingstone et al., 1984; Quinn et al., 1979).

Short-term memory in flies corresponds to a memory with rapid onset and short perdurance (less than 60 min). *dunce* and *rutabaga* mutants are impaired in short-term memory (Chen et al., 1986; Davis and Kiger, 1981; Dudai et al., 1976; Han et al., 1992; Levin et al., 1992; Livingstone et al., 1984; Tempel et al., 1984). *Dunce* encodes a cAMP-specific phosphodiesterase (Chen et al., 1986) and *rutabaga* encodes a calcium-sensitive adenylyl cyclase (AC) (Levin et al., 1992), thus implicating the cyclic adenosine monophosphate (cAMP) pathway in memory formation. Interestingly, neither *rutabaga* mutants nor mutants for the downstream protein-kinase A (PKA) show a complete loss of memory (Han et al., 1992), arguing for the presence of alternative pathways. One such a pathway has recently been identified. *Gilgamesh* (*gish*) encodes a casein kinase I γ

homolog that was recently shown to be involved in short-term memory (Tan et al., 2010). A third pathway involving *turnip*, a protein kinase C (PKC) pathway component, might also be involved (Choi et al., 1991; Mihalek et al., 1997; Tully and Quinn, 1985b).

Less is known about mid-term memory, which can last for up to few hours. However, mid-term memory can be genetically dissected. The *amnesiac* mutant disrupts mid-term memory (Quinn et al., 1979). What exactly *amnesiac* encodes is not entirely clear, but it might be a pre-pro-neuropeptide that has limited homology to the mammalian neuropeptide/hormone pituitary adenylyl cyclase-activating peptide (PACAP) (Vaudry et al., 2000). Further evidence for mid-term memory comes from mutations in DCO, the catalytic subunit of cAMP-dependent protein kinase (PKA) (Skoulakis et al., 1993). A temperature sensitive mutation of DCO showed similar phenotypes to the *amnesiac* mutants (Li et al., 1996).

Long-term memory was originally defined by five criteria: 1) long persistence, 2) dependence on spaced training, 3) resistance to disruption by anesthesia, electroconvulsive shock, cooling, concussion, or other agents that interfere with patterned neural activity, 4) requirement for new protein synthesis, and 5) dependence on the cAMP response-element binding protein (CREB) transcription factor (Bailey et al., 1996). In flies, however, long-term memory has been subdivided into anesthesia-resistant memory and long-term memory. In contrast to long-term memory, anesthesia-resistant memory is resistant to treatment with transcriptional or translational blockers (Isabel et al., 2004; Tully et al., 1994). Anesthesia-resistant memory formation is lost in a *radish* mutant (Folkers et al., 1993), which encodes a protein with 23 predicted cyclic-AMP-dependent protein kinase phosphorylation sequences (Folkers et al., 2006). The PKC pathway is likely also involved in anesthesia-resistant memory. Overexpression of a constitutively active form of aPKC ζ enhances memory in wild-type flies and also improves the memory of *radish* mutants, suggesting that PKC acts downstream of *radish* (Drier et al., 2002). Anesthesia-resistant memory can be distinguished from long-term memory by the way the memories are generated. Massed training (ten training sessions administered one immediately

after the other) only results in anesthesia-resistant memory without long-term memory (Tully et al., 1994). By contrast, spaced training (ten training sessions with breaks in between) produces a long-term memory that consists of two experimentally separable portions. One half is contributed by anesthesia-resistant memory. The other half, long-term memory, can be abolished by inhibiting protein synthesis (with hexamidine) and can last for up to seven days. Long-term memory is not only sensitive to treatment with translational blockers but is also critically dependent on the transcription factor cAMP-response-element-binding protein (CREB) (Tully et al., 1994; Yin et al., 1994). Induced overexpression of CREB repressor completely abolishes long-term memory (Yin et al., 1994).

Why are there two alternative forms of consolidated memory in *Drosophila*? A reason might be the relatively high cost of a translational dependent consolidation. From this perspective it might make sense to have an alternative way of storing information over a longer period while at the same time freeing resources for other tasks, i.e mating. This line of argumentation fits a recent finding that social interactions facilitate anesthesia-resistant memory formation (Chabaud et al., 2009). By contrast, long-term memory is improved when flies are trained individually rather than in a group (Chabaud et al., 2009).

1.1.4 Local protein synthesis in long-term memory formation

The main difference between long-term memory and all other memory phases is the critical requirement for protein synthesis. The hypothesis that long-term memory formation may depend on the production of new proteins was first suggested more than half a century ago (Halstead, 1950). Almost 50 years ago, it was experimentally demonstrated that memory formation in mammals requires protein synthesis (Flexner et al., 1963). The proteins required for the establishment of long-term memory are proposed to stabilize modifications in synaptic strength (Bailey et al., 1992; Glanzman et al., 1990). Until recently it was believed that all proteins required for neuronal function were made in the cell

body. However, experiments in mice and *Aplysia* demonstrated that proteins could be locally translated at synapses (Martin et al., 1997; Mayford et al., 1996).

Despite the experimental validation coming only quite recently (Kang and Schuman, 1996), there were several hints along the way that local translation might be happening. In 1982, polyribosomes were detected beneath postsynaptic sites on the dendrites of central nervous system (CNS) neurons (Steward and Levy, 1982). These experiments led to the speculation that local protein synthesis in dendrites was necessary for local changes at specific synapses, (Steward and Fass, 1983; Steward and Levy, 1982). Later, isolation of synaptosomes, synapses biochemically fractionated from the cell body, provided evidence for local translation in synapses (Feig and Lipton, 1993; Ouyang et al., 1999; Rao and Steward, 1991; Weiler and Greenough, 1991; Weiler et al., 1997). However, it was only in 1996 that a true functional role for local protein translation was found— specifically the local response to the neurotrophin BDNF (Kang and Schuman, 1996). This local protein translation was linked to long-term potentiation in this and subsequent studies from *Aplysia* (Frey and Morris, 1997; Kang and Schuman, 1996; Martin et al., 1997).

1.1.5 Signal transduction pathways leading to protein synthesis

The fact that long-term memory is dependent on synthesis of new proteins requires synapses to communicate this need for proteins to the nucleus. In doing so, the short-term modifications at the synapse are converted to long lasting changes.

The short-term modifications happen as a result of the co-incident detection of a reinforcement signal (probably mediated by dopamine, see above section) and a second cue (in case of courtship conditioning probably pheromone detection) (Baxter and Byrne, 1990; Byrne and Kandel, 1996; Klein et al., 1982). At a molecular level, the pairing of these cues is thought to activate NMDA receptors by release of the magnesium block, which in turns allows influx of calcium into the post-synaptic cell (Dingledine et al., 1999; Liu and Zhang, 2000).

NMDA receptors, in both vertebrates and *Drosophila*, have been implicated in learning and memory (Wu et al., 2007; Xia et al., 2005).

Numerous pathways then make use of the increased calcium as second messenger. One of these pathways is the cyclic adenosine 3,5'-monophosphate pathway (Dash et al., 1990; Schacher et al., 1988)(Fig.3). The above mentioned short-term memory mutants *dunce* and *rutabaga* are involved in this pathway. *Dunce* encodes a cAMP- specific phosphodiesterase (PDE) (Chen et al., 1986; Davis and Kiger, 1981) and *rutabaga* encodes a calcium-sensitive adenylyl cyclase (Levin et al., 1992). In this pathway increased calcium activates the catalytic and regulatory subunit of cAMP- dependent protein kinase A, the primary downstream target of cAMP induction (Drain et al., 1991). Interestingly, two recent studies report enrichment of components of the cAMP pathway in MB axons (lobes). Pairing of the reinforcement signal, dopamine, with depolarization, induced through either shock (Tomchik and Davis, 2009) or acetylcholine (Gervasi et al., 2010), led to elevated cAMP signal mediated through *rutabaga*. When either of the signals was presented on its own no increase in cAMP was observed. This suggests *rutabaga* might act as a co-incidence detector to integrate reinforcement and depolarization signaling (Gervasi et al., 2010; Tomchik and Davis, 2009; Zars et al., 2000).

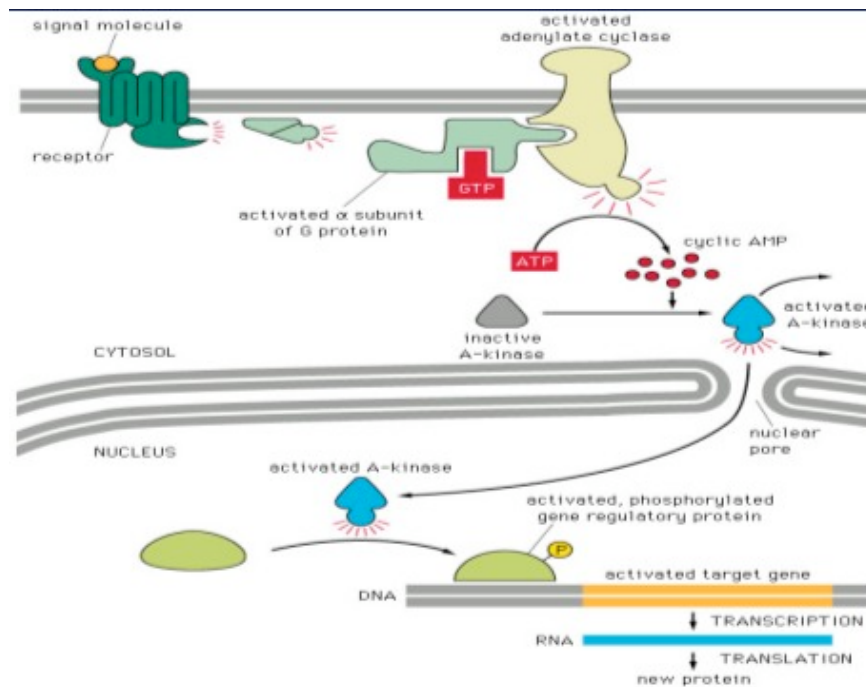


Figure 3 the cAMP pathway

See text for further details

Further downstream of this pathway A-kinase anchoring proteins (AKAPs), help the catalytic subunit of PKA to translocate into the nucleus (Lu et al., 2007). There, PKA activates the transcription factor CREB (Esteban et al., 2003). CREB binds to the cAMP-response-element (CRE) regulating downstream genes leading to synthesis of new mRNA and proteins (Camarota et al., 2000). Heat shock induction of a dominant- negative CREB transgene impaired the acquisition of long-term memory, but left short-term memory unaffected (Yin et al. 1994). CREB is therefore likely to be a key player in protein synthesis dependent memory and potentially the link that enables transition from short- to long-term memory. However, the evidence for CREB being the central transcriptional activator in long- term memory is controversial (Perazzona et al., 2004). Indeed, other signal transduction pathways like the Notch pathway (Ge et al., 2004; Presente et al., 2004) or the Ras/Raf/MAPK pathway (Moressis et al., 2009), as well as some of its downstream components (Matsuno et al., 2009), have also been implicated in long-term memory.

The above pathways contain components that are conserved in vertebrates and invertebrates. However, in *Drosophila* an additional component may also be involved, namely the ecdysozoa specific steroid hormone ecdysone (Ishimoto et al., 2009). In male flies, the levels of 20-hydroxyecdysone (20E), the active metabolite of ecdysone was found to be significantly increased after courtship conditioning, and exogenous administration of 20E either enhanced or suppressed courtship LTM, depending on the timing of its administration (Ishimoto et al., 2009). Mutants in which ecdysone signaling is reduced were defective in long-term memory, and elevation of 20E levels was associated with activation of the cAMP response element binding protein (CREB) (Ishimoto et al., 2009). CREB was therefore suggested to act downstream of ecdysone.

1.1.6 Transport of mRNAs

Signaling via CREB is likely to be the molecular pathway whereby synapse activation leads to new transcription in the nucleus. After transcription, however, the mRNAs need to be transported back to the synapses for local translation to occur. Experiments in *Drosophila* embryos demonstrated that mRNAs can be transported in an inactive state to the place of translation. The 3'UTRs of the mRNAs were shown to control both the transport as well as the translation itself (Driever and Nusslein-Volhard, 1988; Gavis and Lehmann, 1994; Lantz et al., 1992).

In theory, mRNAs could be transported to synapses in one of two ways: 1) the mRNA could just randomly diffuse throughout the entire cell or 2) the mRNA could be actively transported to sub-cellular compartments. Evidence that the latter is happening stems from several observations. First, kinetic analysis of mRNA transport suggested movements compatible with active transport and not diffusion (Lipshitz, 2009; Spirov et al., 2009). Second, sequences within the mRNA determine their final destination. These localization elements (LEs), or zipcodes, determine the location within a cell that the mRNA will be targeted (Mayford et al., 1996; Mori et al., 2000; Rehbein et al., 2000; Tiruchinapalli et al.,

2003). LEs are recognized by RNA binding proteins and together then form ribonucleotide protein (RNP) complexes, which travel along cytoskeleton filaments to the synapse with the help of motor proteins (Aakalu et al., 2001). Some of the RNA binding proteins involved in translational control and long-term memory, like dFMR1, staufer, pumilio, as well as other proteins involved in localization like oscar and eIF4E have been shown to be contained in these structures (Barbee et al., 2006; Ferrari et al., 2007). RNA-containing staufer protein particles move along microtubules in the dendrites (Kiebler et al., 1999; Kohrmann et al., 1999). Expression of a truncated staufer, lacking the microtubule-binding domain, decreases the amount of RNA detected in dendrites (Tang et al., 2001). It remains to be seen whether such localization patterns are required for synapse-specific processes, though initial data would support the idea of such a function (Ferrari et al., 2007). In the next section, I will discuss the function of these RNA binding proteins and how they regulate mRNA translation.

1.1.7 Translational control of transported mRNA

An important question is how mRNAs are translationally silenced during their transport and how this repression is relieved at their destination. It is clear that both, repression and activation need to be tightly controlled in space and time. It is believed that this regulation occurs at the level of translation initiation (Raught et al., 2000).

Translation initiation factors orchestrate the step of ribosomal recruitment to the mRNA 5' cap (Busch et al., 1976). The cap binding factor eIF4E recruits the large ribosomal subunit through interaction with eIF4G. The translational activity of eIF4E is regulated through specific phosphorylation and through its association with inhibitory eIF4E binding proteins 4E-BP1/2 (Gingras et al., 1999). Translation is accomplished through modulation of the activity of the translation initiation factors eIF4E and 4E-BP1/2 (Gingras et al., 2001; Herbert et al., 2002). How does the machinery used in long-term memory interact with this general pathway?

Some RNA binding proteins, implicated in long-term memory, like Pumilio, Staufen, FMRP or CPEBs can control translation in a sequence specific way by binding to specific sequences called recognition motifs within the 3'UTRs of their target mRNAs (Mayford et al., 1996; Mori et al., 2000; Rehbein et al., 2000; Tiruchinapalli et al., 2003). The sequence specific binding might link specific substrates to translational control, thus allowing for the general translational control machinery to be adapted to the more specialized role in long-term memory.

Two distinct mechanisms for mRNA recognition have been proposed for FMRP, the first involving direct mRNA binding mediated by G-quartet structures in target mRNAs (Darnell et al., 2001), and the second involving indirect binding to target mRNAs mediated by the noncoding dendritic RNA BC1 (Zalfa et al., 2003). The BC1-dependent association of FMRP with several dendritic mRNAs, including α CaMKII and Arc, appears to result in their translational repression (Zalfa et al., 2003).

Perhaps the clearest example of translational control in LTM comes from the regulation α CaMKII. The α CaMKII 3' UTR contains a pair of consensus cytoplasmic polyadenylation element (CPE) sequences, which have been implicated in neuronal activity-dependent polyadenylation (Richter and Lorenz, 2002; Wu et al., 1998). Activation of NMDA receptors induces phosphorylation of CPEB leading to increased polyadenylation of the CaMKII 3'UTR (Wells et al., 2001). This increased polyadenylation correlates with an increase in CaMKII levels (Bagni et al., 2000; Ouyang et al., 1999). In addition to the CPE, the 3' UTR of CaMKII is predicted to contain other recognition motifs as well as miRNA binding sites (Mori et al., 2000). This suggests a concerted regulation by more than one factor. Consistent with such an idea, a recent study demonstrated a combinatorial code in which the CPE and the Pumilio-binding element together determine the extent and timing of cytoplasmic polyadenylation-dependent translational activation mediated by CPEB (Pique et al., 2008). Identifying and understanding the interactions between regulators and their target RNAs would be of great benefit in understanding the control of local translation.

1.1.8 Synaptic modifications based on protein synthesis dependent memory

In theory protein synthesis dependent memory formation could modify synapses in one of two ways: 1) structurally 2) or functionally.

Structural modifications of synapses and reorganization of neuronal circuits have been linked to memory storage. Activity dependent reorganization leads to morphological modification of synaptic spines (Becker et al., 2008; Hofer et al., 2009). In *Drosophila*, deprivation of sensory input to the mushroom body leads to a long-lasting modification of the calyx, the dendritic area of the mushroom body (Kremer et al., 2011). Further evidence for structural rearrangements comes from the finding that manipulations of ras/Raf/MAPK pathway interfere with both dendritic spine morphology and long-term memory (Billuart and Chelly, 2003; Schenck et al., 2003). The ras/Raf/MAPK pathway is well known to be involved in actin-dependent cellular remodeling (Bustelo et al., 2007). However, this pathway is not only involved in stabilization of memory but also in forgetting. A recent study reported that inhibition of Rac activity leads to slower decay of early memory, extending it from a few hours to more than one day. Conversely, Rac elevation leads to accelerated interference-induced forgetting (Shuai et al., 2010). Together, this data further strengthens the idea that structural changes at the synapse, i.e mediated through actin cytoskeleton remodeling may contribute to memory formation and erasure. In some cases structural changes have been shown to be dependent on protein synthesis and degradation (Fonseca et al., 2006). A number of proteases have been implicated in LTM (Dash et al., 2000). *Tequila*, encoding a serine protease (Sonderegger and Patthy, 2007), and *crammer*, encoding a cysteine protease inhibitor of the cathepsin family (Comas et al., 2004), are the best examples of proteases that might be involved in long-term memory. The observation that both proteases and protease inhibitors are found to alter memory argues that both protein stabilization and degradation are important in memory formation.

One of the functional modifications, and probably a key element in the process of increasing the synaptic efficiency, is the incorporation of additional

AMPA receptors into the post-synaptic density (Esteban et al., 2003; Hayashi et al., 2000). As a consequence this leads to increased excitability, which in turn can lead to the phenomena of long-term potentiation, the underlying basis for long-term memory (Rumpel et al., 2005).

1.1.9 Local translation might not be sufficient to explain synapse specific modifications

As described above, long-term memory requires new protein synthesis at specific synapses. In theory local protein synthesis might achieve such synapse specificity, albeit it would require the proteins or mRNAs to be transported selectively to specific synapses. An alternative mechanism for achieving synapse specific strengthening during long-term memory might be “synaptic tagging”. This hypothesis suggests that proteins or mRNAs are available to all the synapses of a neuron but are selectively captured by activated synapses.

Recent work on the bifurcated Schaeffer collateral pathway, where two inputs converge on the same cell suggests the existence of such a “synaptic tag”, which primes the activated synapse as stimulated for long-term modifications (Frey and Morris, 1997).

Since applying protein-synthesis inhibitors specifically during the stimulation still resulted in long-term changes, it was further suggested that the first step of synaptic ‘tagging’ did not require protein synthesis (Frey and Morris, 1997; Martin et al., 1997). Although local protein synthesis is not needed for the ‘tagging’ itself, it is required for the stable maintenance of the synaptic changes (Casadio et al., 1999). Thus, the nature of the tag might have two components: a protein synthesis independent early step, and a transcription and protein synthesis dependent late component. The latter component requires translation of some mRNAs locally at the synapse suggesting that these mRNAs may be dormant before they reach the site of translation. This would suggest the tag might consist of a translational regulator that is capable of localizing mRNA to synapses and allow local translation. Although a number of candidates have been suggested

for the tag (Frey and Morris, 1998; Martin and Kosik, 2002), its exact nature remains elusive.

One candidate that might fit both components is a family of proteins called Cytoplasmic Polyadenylation Element Binding proteins (CPEBs). CPEBs have been proposed to contain domains that act in a prionic fashion (Heinrich and Lindquist, 2011; Si et al., 2003b). Prions are proteins that can self-catalyze themselves to a 'prion state'— an alternative folding state of the protein. Historically, it was thought that this 'prion state' resulted in loss of function of the protein due to the formation of large aggregates. The 'prionic state' is thought to be quite stable and possibly irreversible. As evidence, a yeast prion is able to perpetuate its prionic state through many generations (Patino et al., 1996; Paushkin et al., 1997; Sparrer et al., 2000; Wickner, 1994; Wickner et al., 1995). However, subtler positive roles have now been suggested for the 'prionic state'. CPEBs are thought to be able to switch and maintain a change in structure in a protein synthesis independent manner, which might mark the synapse in a long-lasting way (Si et al., 2003a; Si et al., 2003b). CPEBs, therefore meet the first criteria. Secondly, CPEBs are well known to control local translation (Hake and Richter, 1994). Therefore, they can also satisfy the second criteria.

1.1.10 Translational control by the CPEB1 subfamily

Members of the CPEB family proteins all carry an RNA binding domain (RBD) at their C-terminus, consisting of two RNA recognition motifs (RRM), and a Zinc finger motif (Znf) (Fig.4) (Hake et al., 1998; Hake and Richter, 1994; Huang et al., 2006). Based on these RBDs CPEB proteins fall into two ancient subfamilies, CPEB classI and classII (Theis et al., 2003) (Fig.4). The overall sequence is not well conserved even within one subfamily (Huang et al., 2006; Mendez and Richter, 2001) However, the RBDs of these proteins are highly conserved within one subfamily but not between the two subfamilies, suggesting different mRNAs are bound by the two families (Fig.4).

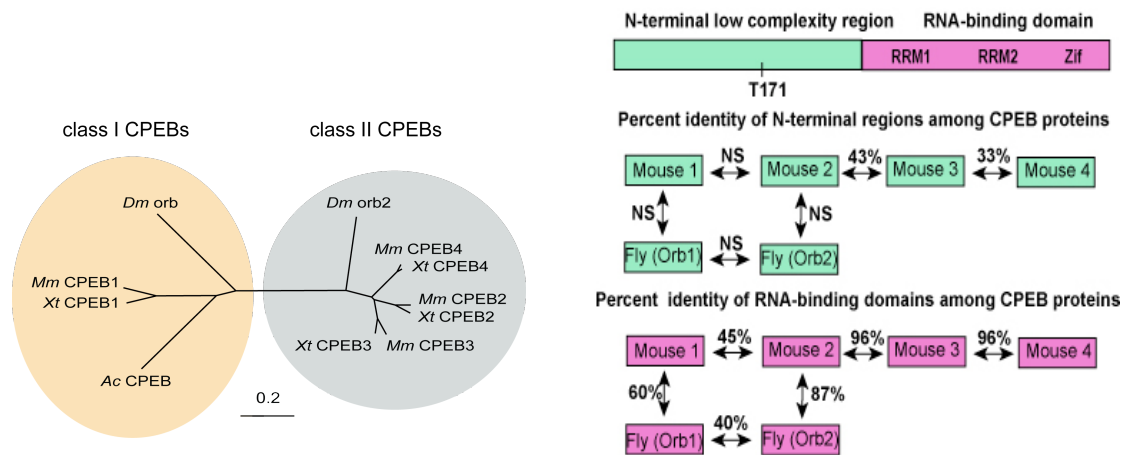


Figure 4 The CPEB protein family

Left: Phylogenetic tree based on an alignment of selected CPEB proteins, showing CPEBs to fall into two ancient subfamilies. Right: All CPEB-like proteins have a carboxy-terminal region containing two RRM domains and a zinc-finger (Znf). Among the amino-terminal regions of the CPEB proteins, there is little identity (NS, not significant). Among the RBDs, there is considerable identity. However, mouse CPEB (designated CPEB1 for convenience) is closer to *Drosophila* CPEB (Orb) than it is to mouse CPEB2; in addition, *Drosophila* CPEB2 (Orb2) is more similar to mouse CPEB2 than it is to Orb. Mouse CPEB2–4 are nearly identical in the RBDs (Huang et al., 2006).

Members of the CPEB1 subfamily (or class I), to which mCPEB1, AcCPEB1 and the *Drosophila* homologue Orb (Lantz et al., 1992) belong, have been shown to localize to synapses (Si et al., 2003a; Wu et al., 1998) and contribute to long-term potentiation (Alarcon et al., 2004) and facilitation (Si et al., 2003a). In addition they have been implicated in various processes like oocyte development (Sheets et al., 1995; Stebbins-Boaz et al., 1996; Tay et al., 2003), cell cycle progression (Groisman et al., 2002; Novoa et al., 2010), and dendrite morphogenesis (Bestman and Cline, 2008, 2009).

Intriguingly, these CPEB proteins have been shown to act as both translational repressor and activator (de Moor and Richter, 1999; Mendez et al., 2000b). In developing *Xenopus laevis* oocytes CPEB activity is regulated by progesterone-induced phosphorylation through Aurora A kinase (Barnard et al., 2004; Mendez et al., 2000a; Mendez et al., 2000b)(Fig.5). In the unphosphorylated state, CPEB is associated with maskin. The CPEB/maskin complex can bind eIF4E. When bound to CPEB/masking, eIF4E is prevented

from recruiting the initiation complex and therefore translation is prevented. Phosphorylation leads to dissociation of maskin, allowing cap binding of eIF4E (Fox et al., 1989; Hake and Richter, 1994) and recruitment of a multiprotein complex containing Gld(2) poly(A)polymerase (Barnard et al., 2004) (Fig.5). This complex further includes the cleavage and polyadenylation specificity factor (CPSF), which binds the hexanucleotide AAUAAA, another cis element in the RNA essential for polyadenylation, symplekin, a scaffold protein that helps to link CPEB to CPSF, guanine nucleotide exchange factor (Reverte et al., 2003), an RNA helicase (Minshall and Standart, 2004), and amyloid precursor proteins (Cao et al., 2005)(Fig.5). All these factors help Gld(2) to polyadenylate mRNA which allows the mRNA translation to start. It is this direct control of polyadenylation, which makes CPEB proteins unique in translational control. It is the regulation of the polyA length that allows them to be translational inhibitor and translational activator at the same time (de Moor and Richter, 1999; Mendez et al., 2000b).

CPEB proteins of the class I control translation by binding to short canonical sequences called cytoplasmic polyadenylation elements (CPE elements with a consensus sequence U₄₋₅A₁₋₃U) in the 3'UTRs of their target RNAs (Fox et al., 1989). Both RRM and the ZnF domains are required for high affinity binding to CPE elements (Hake et al., 1998)

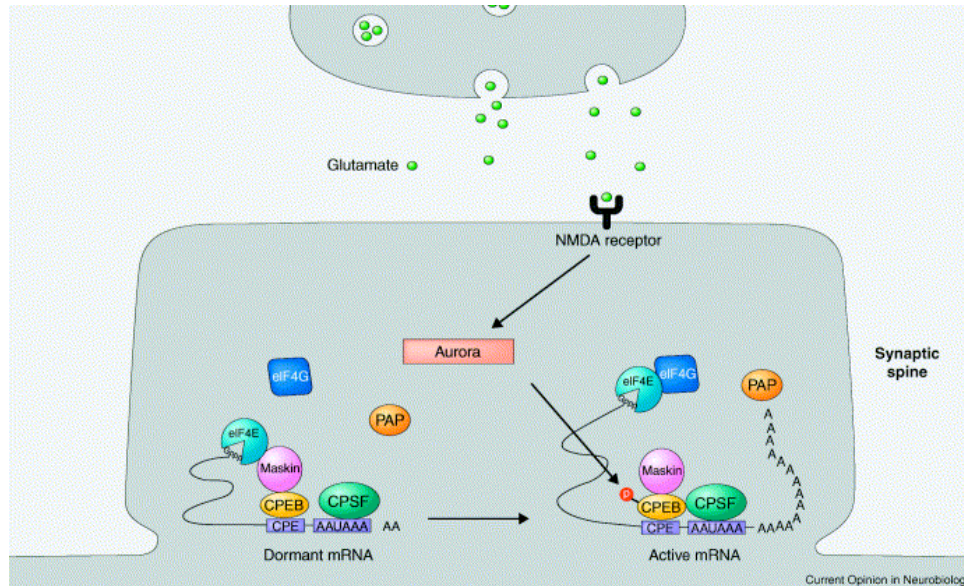


Figure 5 Translational control of mRNA by CPEB class I proteins

Following NMDA receptor activation by neurotransmitter (glutamate), the protein kinase aurora becomes activated and phosphorylates CPEB, the CPE-interacting factor. This modification probably helps CPEB stabilize CPSF on the AAUAAA hexanucleotide, which in turn attracts PAP to the end of the mRNA where it catalyzes polyadenylation. Maskin, a CPEB-associated protein, then dissociates from eIF4E, the cap binding factor, allowing eIF4G to bind eIF4E and initiate translation (Richter and Lorenz, 2002).

1.1.11 The CPEB2-4 subfamily

Members of the second, more recently identified class of CPEB proteins (mCPEB2-4 or class II) (Mendez et al., 2002; Sheets et al., 1995; Theis et al., 2003), containing the vertebrate mCPEB2, 3 and 4, are also expressed in the brain, where they localize to post-synaptic densities (Huang et al., 2006). Orb2 is the *Drosophila* homologue of the mCPEB2-4 subfamily and is predicted to be a neuronal gene based on *in situ* hybridization experiments (Keleman et al., 2007; Si et al., 2003a). Like the CPEBI family, CPEBII family members are also associated with RNPs (Bestman and Cline, 2009; Chae et al., 2010; Cziko et al., 2009). Orb2 is localized to granular structures and co-localizes with the RNP component FMR1 (Cziko et al., 2009). However, a direct interaction between these two proteins has not yet been demonstrated (Kwak et al., 2008).

Similar to the CPEB1 subfamily, CPEB2-4 proteins are also involved in many different processes including cell cycle progression (Novoa et al., 2010) and transcription (Peng et al., 2010). Members of the CPEB2-4 subfamily also control translation of mRNA, but accomplish this by a so far unknown mechanism, which involves neither polyadenylation nor binding of CPE elements (Huang et al., 2006). It has been suggested that CPEB2-4 might recognize tertiary structures within the 3'UTR of their target mRNAs (Huang et al., 2006).

However, while CPEB protein are involved in translational control of many mRNAs, also translation of CPEB mRNA itself is tightly controlled.

1.1.12 Translational regulation of CPEB proteins

CPEB2-4 mRNAs are predicted to be regulated by a number of miRNAs as well as other RNA binding proteins, however their exact function needs to be determined (Morgan et al., 2010). By date the precise CPEB target mRNAs remain largely unknown although in the case of Orb2 some have been recently suggested (Mastushita-Sakai et al., 2010). These involve coding sequences for proteins required for various processes like neuronal growth, synapse formation, and protein turnover (Mastushita-Sakai et al., 2010). Interestingly, this data suggested that one of the Orb2 mRNA targets is its own mRNA. Thus Orb2 might control its own translation (Mastushita-Sakai et al., 2010), implying a feedback loop.

Similar findings were made in a recent work in mice. There CPEB proteins can control translation of CPEB mRNA of homologous CPEBs (Igea and Mendez, 2010; Novoa et al., 2010). Such a feed back loop might be used in synaptic tagging where the tag facilitates its own translation, thus amplifying an initially weak signal.

1.1.13 CPEB as a candidate for the synaptic tag

How do long-term memories remain stable if the proteins required to maintain them are exposed to constant turnover? A possible solution lies in self-sustaining properties of the proteins required to stabilize the memory.

The ability of CPEBs to control translation of target mRNAs like CaMKII locally at synapses (Du and Richter, 2005) made them interesting candidates for a synaptic tag. However, behavioral tests on CPEB1 knockout mice did not show the expected phenotype in long-term memory, but showed only a role in memory extinction (Berger-Sweeney et al., 2006). It therefore seems unlikely that members of the CPEB1 family constitute the synaptic tag required for long-term memory. However, during the last decade, other members of the Cytoplasmic Polyadenylation Element Binding proteins (CPEB) family proteins, the *Aplysia* CPEB1 (Miniaci et al., 2008; Si et al., 2010; Si et al., 2003a; Si et al., 2003b) and the *Drosophila* homologue Orb2 (Keleman et al., 2007), have been suggested to function as a synaptic tag.

In *Aplysia* CPEB1 lacks the consensus phosphorylation site for Aurora kinase, required for activation of other CPEBs (Mendez et al., 2000a); however, it contains a low complexity region in the N-terminus that is enriched in glutamine content. In *Aplysia*, this Q-rich domain has been proposed to be involved in activity regulated translational control (Si et al., 2010; Si et al., 2003a; Si et al., 2003b). This domain shows close similarity to yeast prionic domains (Heinrich and Lindquist, 2011; Si et al., 2003b). Surprisingly, in the case of the AcCPEB1, it is the self-perpetuating prion-like form that has the greatest capacity to stimulate translation (Si et al., 2010). As with all other prions, the observed aggregates of AcCPEB1 were shown to consist of homotypic interaction of CPEB, which have amyloidogenic and self-sustaining properties (Heinrich and Lindquist, 2011; Si et al., 2010). When tested functionally, an antibody that preferentially recognizes the multimeric state selectively inhibits the persistence of long-term facilitation of sensory-motor neuron synapse (Si et al., 2010). Interestingly such a mode of regulation might exist outside of the CPEB family.

The Pumilio protein, implicated in long-term memory (Dubnau et al., 2003), also contains a Q-domain that can aggregate in yeast and shows amyloidogenic self-sustaining properties (Salazar et al., 2010). However, it should be noted that so far no aggregation has been seen *in vivo* upon synaptic stimulation, nor has aggregation *in vivo* been directly functionally linked to memory, thus many of these models remain controversial at best.

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2. Function of the *Drosophila* CPEB protein Orb2 in long-term memory

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Both long-term behavioral memory and synaptic plasticity require protein synthesis, some of which may occur locally at specific synapses. Cytoplasmic polyadenylation element-binding (CPEB) proteins are thought to contribute to the local protein synthesis that underlies long-term changes in synaptic efficacy, but a role has not been established for them in the formation of long-term behavioral memory. We found that the *Drosophila melanogaster* CPEB protein Orb2 is acutely required for long-term conditioning of male courtship behavior. Deletion of the N-terminal glutamine-rich region of Orb2 resulted in flies that were impaired in their ability to form long-term, but not short-term, memory. Memory was restored by expressing Orb2 selectively in fruitless (*fru*)-positive γ neurons of the mushroom bodies and by providing Orb2 function in mushroom bodies only during and shortly after training. Our data thus demonstrate that a CPEB protein is important in long-term memory and map the molecular, spatial and temporal requirements for its function in memory formation.

2.1 Introduction

Both long-term behavioral memory and synaptic plasticity require protein synthesis, some of which may occur locally at specific synapses. Cytoplasmic polyadenylation element-binding (CPEB) proteins are thought to contribute to the local protein synthesis that underlies long-term changes in synaptic efficacy, but a role has not been established for them in the formation of long-term behavioral memory. We found that the *Drosophila melanogaster* CPEB protein Orb2 is acutely required for long-term conditioning of male courtship behavior. Deletion of the N-terminal glutamine-rich region of Orb2 resulted in flies that were

impaired in their ability to form long-term, but not short-term, memory. Memory was restored by expressing Orb2 selectively in fruitless (*fru*)-positive γ neurons of the mushroom bodies and by providing Orb2 function in mushroom bodies only during and shortly after training. Our data thus demonstrate that a CPEB protein is important in long-term memory and map the molecular, spatial and temporal requirements for its function in memory formation.

Long-term memory is thought to reflect experience-dependent morphological and biochemical changes at specific neuronal synapses. Because these changes rely on the selective utilization of mRNAs that are potentially available to all synapses^(1, 2,) it has been postulated that stimulated synapses establish a synaptic tag that marks them as sites for the local synthesis⁽³⁾ or capture⁽⁴⁾ of the relevant proteins. The molecular nature of this synaptic tag is unknown. A critical feature for any component of a synaptic tag is that it should be required for long-term behavioral memory, but should be dispensable for memory acquisition, short-term maintenance and recall. Additionally, the tag should be required in the relevant neural circuits, and only during or shortly after training. We show here that the *Drosophila* CPEB protein Orb2 meets all of these requirements.

CPEB proteins fall into two ancient subfamilies (Fig. 1a). The CPEB1 subfamily includes *Xenopus* CPEB5 and its *Drosophila* ortholog Orb6, both of which function in the regulation of mRNA translation during oogenesis^(5, 7, 8, 9, 10, 11). These proteins bind cytoplasmic polyadenylation elements in the 3' untranslated regions of dormant cytoplasmic mRNAs, triggering their polyadenylation and translation^(5, 7). The *Aplysia californica* and mouse CPEB1 proteins are also localized at synapses^(12, 13) and contribute to long-term facilitation and potentiation, respectively^(13, 14). These observations have led to speculation that CPEB1 proteins might be components of the synaptic tag. However, extensive behavioral tests on CPEB1 knockout mice have revealed defects in memory extinction, but have failed to provide any evidence for a role of CPEB1 in long-term memory formation⁽¹⁵⁾.

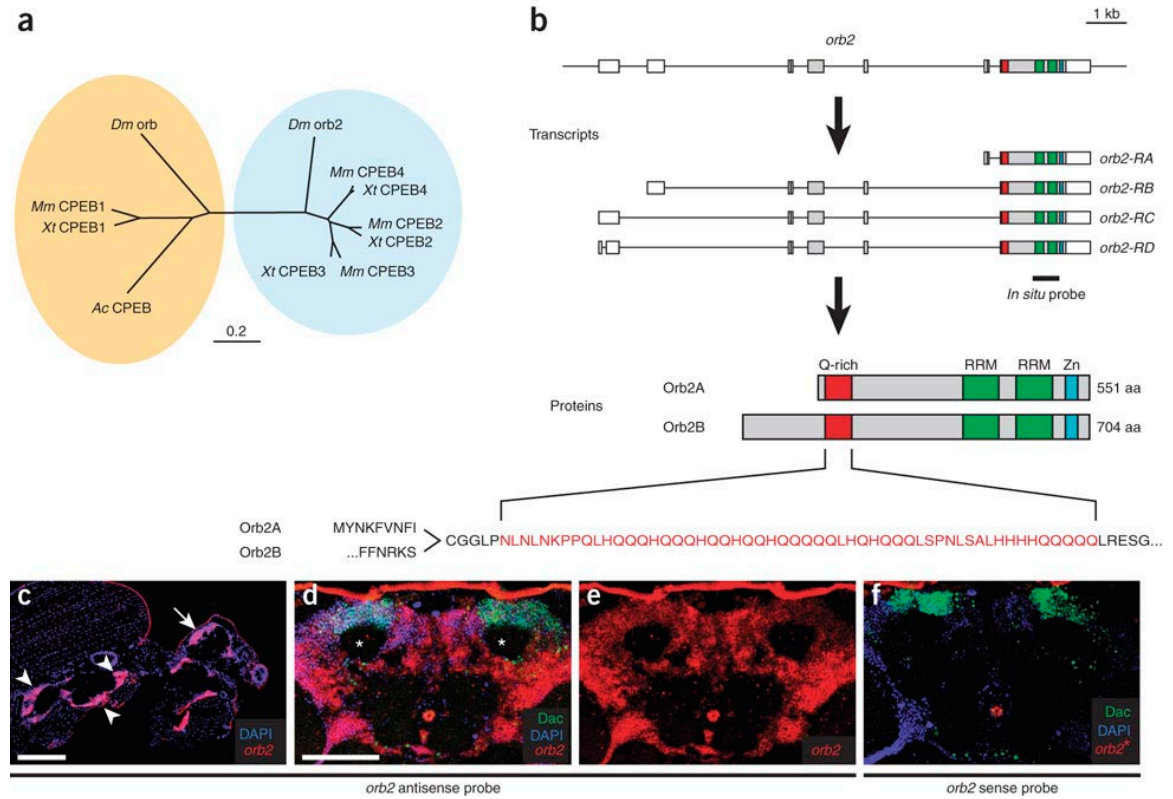


Figure 1: Structure and expression of *Drosophila orb2*.

(a) Unrooted phylogenetic tree based on an alignment of selected CPEB proteins. Scale indicates observed sequence divergence. Ac, *Aplysia californica*; Dm, *Drosophila melanogaster*; Mm, *Mus musculus*; Xt, *Xenopus tropicalis*. (b) Organization of the *orb2* genomic locus and transcripts, and predicted Orb2 proteins. aa, amino acids; Q-rich, glutamine-rich region; Zn, zinc-finger motif. The sequence spanning the Q-rich region is shown, with red indicating the amino acids that are deleted in the *orb2Q* allele. (c) Sagittal section of a wild-type fly hybridized with an antisense *orb2* probe to detect all isoforms (red) and counterstained with DAPI (blue). Note the prominent *orb2* expression in the brain (arrow) and ventral ganglia (arrowheads). Scale bar, 100 μ m. (d,e) Transverse section of an adult head, hybridized with an antisense *orb2* probe (red) and counterstained with antibody to Dachshund (Dac) to visualize the nuclei of the mushroom body Kenyon cells (green, d) and DAPI (blue, d). *orb2* mRNA was detected in most cell bodies, but was largely excluded from neuropilar regions such as the mushroom body calyces (asterisks). (f) Head section stained and imaged as in d, but hybridized with the control sense-strand *orb2* probe (red).

Drosophila Orb2 belongs to the CPEB2 subfamily, together with vertebrate CPEB2–4. CPEB2 proteins also stimulate mRNA translation, but evidently do this through a distinct mechanism that involves neither polyadenylation nor the

cytoplasmic polyadenylation element⁽¹⁶⁾. Similar to CPEB1, CPEB3 and CPEB4 are also expressed in the brain and localize to postsynaptic densities, and RNAi knockdown experiments suggest that CPEB3 may regulate levels of the AMPA receptor GluR2⁽¹⁶⁾. Functional studies of CPEB2 subfamily proteins in behavioral learning and memory have not yet been reported. Here, we demonstrate that the *Drosophila* CPEB2 protein Orb2 is acutely required for the formation of a long-term behavioral memory and map this requirement to a specific subset of mushroom body neurons. We suggest that CPEB2 proteins are either components or regulators of the synaptic tag.

2.2 Results

2.2.1 Structure and expression of *Drosophila orb2*

CPEB proteins consist of a highly conserved carboxyl-terminal region that includes two RNA recognition motifs (RRM) and a zinc finger motif. The amino-terminal region is less well conserved, but includes several low-complexity regions with biased amino-acid composition. In *Aplysia* CPEB, this domain acts as a prion-forming domain⁽¹⁷⁾. The *Drosophila orb2* gene produces at least four transcripts (RA, RB, RC and RD) that encode two distinct protein isoforms, Orb2A and Orb2B (Fig. 1b), which share the same RRM and zinc finger motif domains. Both proteins also contain an amino-terminal, glutamine-rich region (red in Fig. 1b), which is similar to the prion-forming domain of *Aplysia* CPEB⁽¹⁷⁾.

We used in situ hybridization to assess the distribution of *orb2* in adult flies (Fig. 1c–f). The *orb2* gene appeared to be a predominantly neuronal gene (Fig. 1c–e), with broad expression throughout the brain and ventral ganglia (Fig. 1c). Expression in the brain included the mushroom bodies (Fig. 1d and ref. 13), structures implicated in learning and memory⁽¹⁸⁾. On the basis of its structure and expression, we considered Orb2 to be a strong candidate for the regulation of long-term memory in *Drosophila*.

2.2.2 Orb2 functions in long-term memory formation

We generated two orb2 mutant alleles by homologous recombination (Fig. 2a and Supplementary Fig. 1 online). In one allele, orb2^Δ, the large 3' exon encoding the RRM and zinc finger motif domains was deleted, and the allele was predicted to be a null allele. The other allele, orb2^{ΔQ}, contained an in-frame deletion of 54 amino acids that specifically removed the glutamine-rich region from both isoforms. We verified the genomic structure of both alleles by PCR and DNA sequencing, and the intended disruptions of orb2 transcripts by RT-PCR (Fig. 2b). The null orb2 mutants were homozygous lethal, but orb2^{ΔQ} homozygotes and orb2^{ΔQ} /orb2^Δ trans-heterozygotes were viable. We did not detect any gross morphological defects in these animals, including their overall brain and mushroom body anatomy (Fig. 2c–e). Both alleles were backcrossed for at least five generations onto a wild-type Canton S background before we carried out any behavioral analyses.

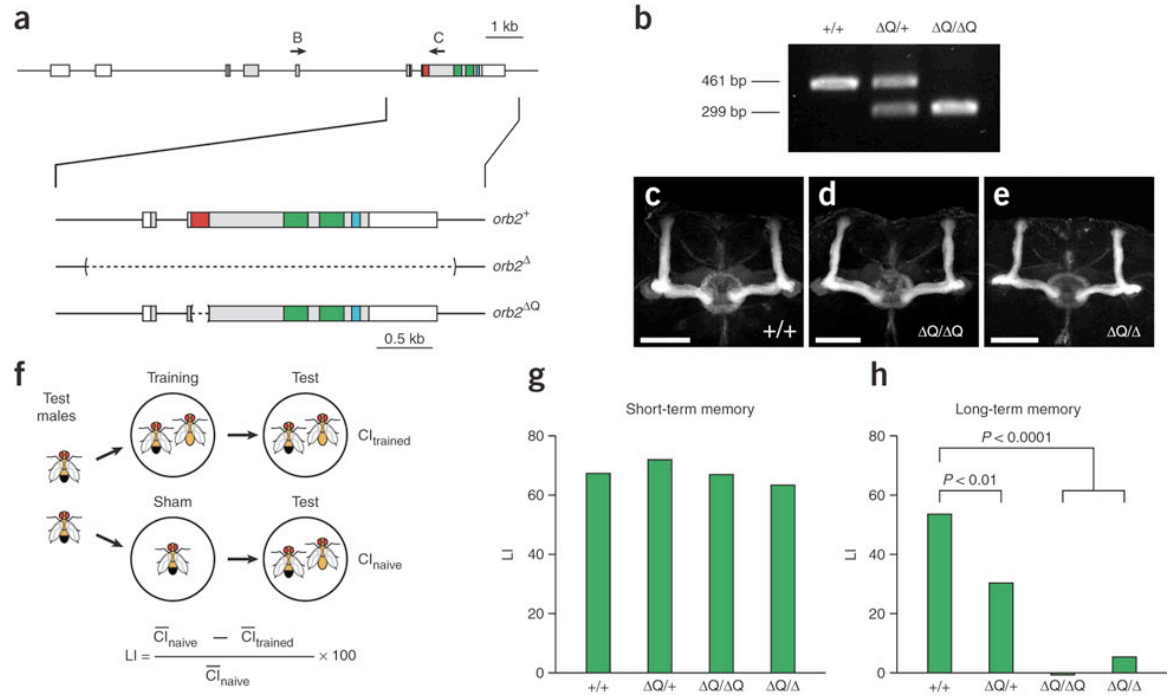


Figure 2: Targeted mutations in *Drosophila orb2* disrupt long-term memory.

(a) Structure of the *orb2*^Δ and *orb2*^{ΔQ} alleles, generated by homologous recombination. Dashed lines indicate deleted regions. (b) RT-PCR products from adult head RNA, amplified using primers B and C indicated in a. Product sizes were as predicted and were verified by DNA sequencing. (c–e) Mushroom bodies of wild-type (+/+, c), *orb2*^{ΔQ}/*orb2*^{ΔQ} (ΔQ/ΔQ), d) and *orb2*^{ΔQ}/*orb2*^Δ (ΔQ/Δ), e) males were stained with antibody to FasII. Loss of *orb2* function did not alter overall mushroom body morphology. Scale bars, 50 μm. (f) Schematic of the courtship conditioning procedure. Males were kept in isolation between eclosion and the training period and between training and testing. The LI was calculated from the mean courtship indices (CIs) as indicated. (g,h) LIs of males carrying the indicated *orb2* alleles, tested with a mated female either for short-term memory 30 min after a 1.5-h training period (g and Supplementary Table 2) or for long-term memory 24 h after a 5-h training period (h and Supplementary Table 4). P values are for H_0 , $LI = LI_{+/+}$ (permutation test).

We tested *orb2*^{ΔQ}/*orb2*^Δ and *orb2*^{ΔQ}/*orb2*^{ΔQ} mutants for learning and memory defects in the context of male courtship behavior, as the neural circuitry that governs this behavior is becoming increasingly well understood^(19, 20). We used the courtship conditioning procedure, in which a male learns to suppress

his courtship activity on the basis of prior exposure to unreceptive mated females^(21, 22, 23, 24) (Fig. 2f). In this procedure, socially naive males are first trained by being individually paired in a courtship chamber with a single mated wild-type female. After a rest period in isolation, these males are then tested for courtship conditioning in subsequent pairings with either an immobilized virgin²¹ or a second mated female^(22, 24, 25). Previous experience with an unreceptive mated female does not suppress subsequent courtship toward a receptive, mobile virgin female (refs. 25–27 and K.K., unpublished data), suggesting that the male learns to better discriminate between receptive and unreceptive females.

To quantify this form of courtship conditioning, the 10-min test assays were videotaped and each assay was subsequently scored blind to both the genotype and the experimental condition. For each male, a courtship index was determined, defined as the percentage of time that the male courts during the 10-min test period. The learning index (LI) was then calculated as the percentage reduction in mean courtship activity of trained males compared with naive males (Fig. 2f).

We first examined short-term memory in *orb2* mutants. A control, wild-type male that was previously exposed for 1 h to a mated female forms a short-term memory that lasts for at least 2–3 h when tested with an immobilized virgin female⁽²¹⁾. Similarly, our control males showed strong courtship suppression when tested 30 min after a 1.5-h training session, using either immobilized virgins (Supplementary Fig. 2 and Supplementary Table 1 online) or mated females (Fig. 2g and Supplementary Table 2 online) as testers. We also observed an identical short-term memory in both *orb2*^{ΔQ} homozygotes and *orb2*^{ΔQ}/*orb2*^Δ trans-heterozygotes. We conclude that courtship behavior itself and short-term memory of courtship conditioning are both intact in *orb2*^{ΔQ} mutants.

A longer 5-h training session produces a long-term memory that lasts for at least 24 h, and may persist for several days⁽²³⁾. Males court on-and-off during this extended training period, which probably mimics the spaced training that is

essential for long-term memory formation in other procedures^(28, 29). Indeed, identical courtship suppression was also obtained with three spaced 1-h training sessions⁽²³⁾. Similarly, when we tested our control males 24 h after a single 5-h training session, we observed a clear courtship suppression using either immobilized virgin or mated female testers (Fig. 2h, Supplementary Fig. 2 and Supplementary Tables 3 and 4 online). In contrast, courtship suppression was completely abolished in both $orb2^{\Delta Q}$ homozygotes and $orb2^{\Delta Q}/orb2^{\Delta}$ trans-heterozygotes, as assayed using either type of tester female (Fig. 2h, Supplementary Fig. 2 and Supplementary Tables 3 and 4). Notably, $orb2^{\Delta Q}/+$ heterozygotes showed an intermediate level of suppression, suggesting that Orb2 levels may be a limiting factor in long-term memory formation (Fig. 2h and Supplementary Table 4). Because assays with mated female testers revealed a more robust long-term memory^(22, 23, 24) (Fig. 2h and Supplementary Fig. 2) and are ecologically more relevant than assays with immobilized virgins^(25, 26, 27,) we used mated females as testers in all subsequent experiments.

2.2.3 Memory does not persist beyond 9 h in orb2 mutants

To assess when the memory of courtship conditioning decays in $orb2^{\Delta Q}/orb2^{\Delta}$ mutants, we determined their courtship activity at various intervals after a 5-h training session (Fig. 3a and Supplementary Table 5 online). We found that memory persisted at its initial levels in $orb2^{\Delta Q}$ mutants for up to 6 h after training, and was indistinguishable from memory levels in the control males during this period. However, although control males retained these initial memory levels for at least 24 h, memory was completely lost in the mutants by 9 h post-training (Fig. 3a and Supplementary Table 5). This time course of memory decay in $orb2^{\Delta Q}$ mutants trained for 5 h matched that observed for wild-type males trained for only 1 h (Fig. 3b and Supplementary Table 6 online). Evidently, in contrast to wild-type males, $orb2^{\Delta Q}$ mutants did not benefit from longer training

sessions, even if training was extended to 8 h (Fig. 3c and Supplementary Table 6).

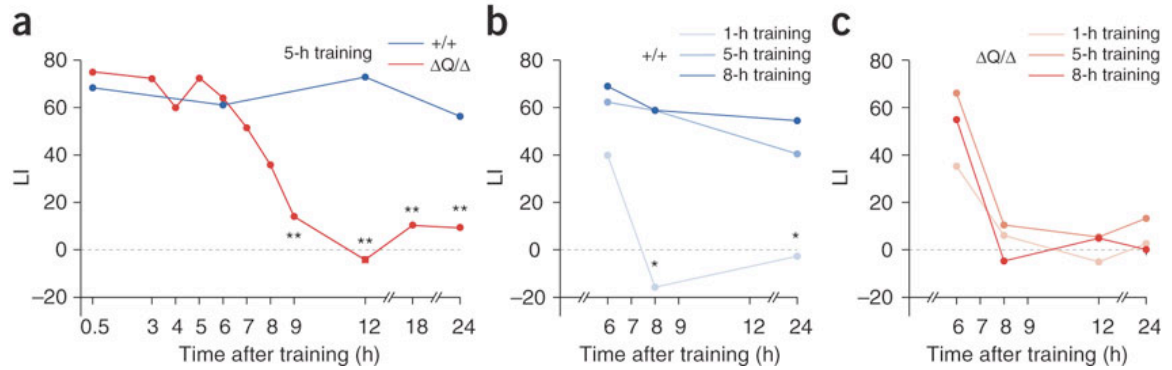


Figure 3: Memory does not persist beyond 9 h in $orb2^{\Delta Q}$ mutants.

LI for $orb2^{\Delta Q}/orb2^{\Delta}$ mutant and wild-type (+/+) control males tested with mated females at various intervals after training, as indicated. (a) Dynamics of memory decay (Supplementary Table 5). ** $P < 0.0001$ for H_0 , $LI = LI_{0.5h}$ (permutation test). (b,c) Effect of training duration on memory persistence in wild-type (b) and $orb2^{\Delta Q}/orb2^{\Delta}$ mutant (c) males (Supplementary Table 6). * $P < 0.06$ for H_0 , $LI = LI_{5h}$ (permutation test).

2.2.4 $orb2$ function is required in mushroom body neurons

Neurons that express the male-specific fru isoforms are required for male courtship behavior^(19, 20,) and it is therefore possible that these are the neurons in which $orb2$ function is required for long-term courtship memory. To test this, we prepared UAS- $orb2A$ and UAS- $orb2B$ transgenes and expressed them in $orb2Q/orb2$ mutants using a fruGAL4 driver²⁰ (Fig. 4a). We obtained full restoration of 24-h memory with both transgenes (Fig. 4 and Supplementary Table 7 online). The driver fruGAL4 is expressed in some 3,000 neurons that are dispersed throughout the nervous system, including a subset of mushroom body intrinsic neurons⁽²⁰⁾. The mushroom bodies are critical for courtship conditioning^(23, 30) and for other forms of olfactory memory^(31, 32, 33, 34,) and fru function is required in these neurons for short-term courtship conditioning⁽¹⁹⁾. We therefore considered the fru-positive mushroom body neurons to be the most

likely site for Orb2 function in long-term courtship conditioning. Consistent with this hypothesis, we were also able to rescue orb2 mutants by expressing the UAS-orb2 transgenes specifically in mushroom bodies, using either of two independent GAL4 drivers³³ (c772-GAL4 and 247-GAL4; Fig. 4b–d and Supplementary Table 7).

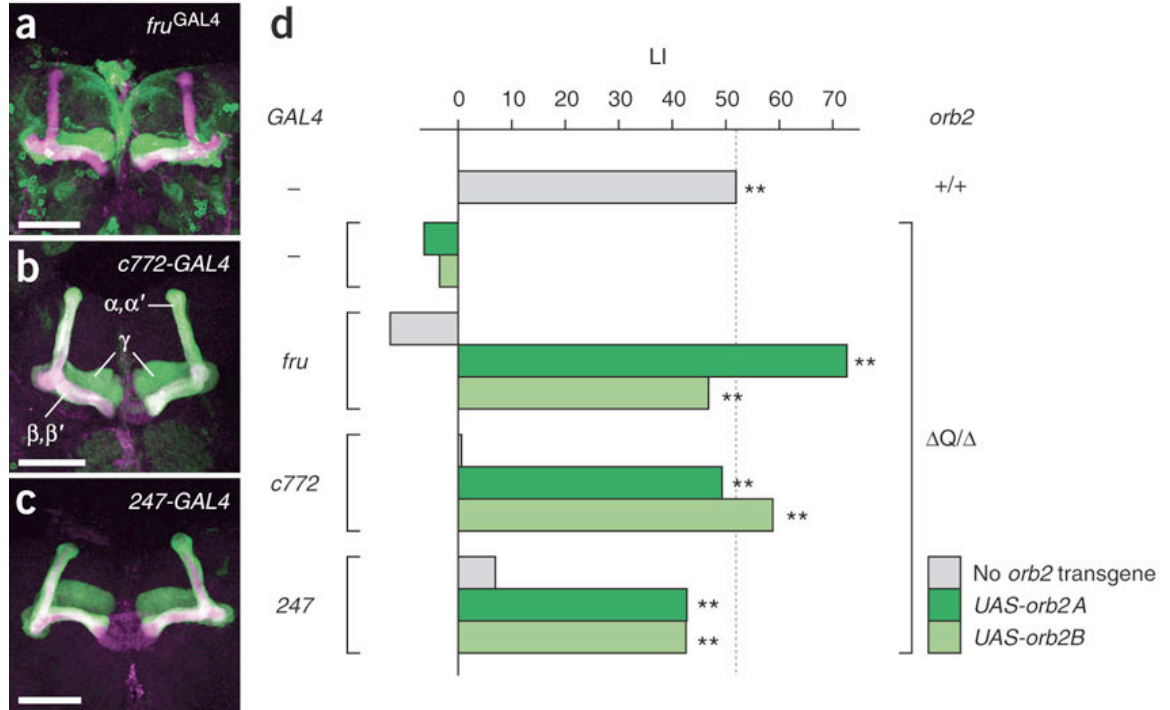


Figure 4: Rescue of long-term memory.

(a–c) Confocal projections through the mushroom body lobes of adults carrying the indicated GAL4 drivers together with UAS-CD8-GFP, stained with antibody to green fluorescent protein (GFP) and counterstained with antibody to FasII (magenta) to visualize the $\alpha\beta$ and γ neurons. Axons of $\alpha\beta$ and $\alpha'\beta'$ neurons bifurcated into vertical and horizontal lobes. Axons of γ neurons did not bifurcate and projected medially in a horizontal lobe that was well separated from the β and β' lobes. Scale bars, 50 μ m. (d) LIs of wild-type (+/+) or orb2^{ΔQ}/orb2^Δ (ΔQ/Δ) males in tests of courtship conditioning performed 24 h after a 5-h training period (Supplementary Table 7). GAL4 drivers were used to express UAS-orb2A or UAS-orb2B in the orb2 mutant background, as indicated (– denotes no GAL4 driver). **P < 0.0001 for H₀, LI = 0 (permutation test).

2.2.5 orb2 function is required during or shortly after training

The long-term memory deficit in orb2^{ΔQ} mutants could reflect either an acute defect in neuronal plasticity or a developmental defect in neuronal wiring. To distinguish between these possibilities, we used the TARGET (temporal and regional gene expression targeting) system⁽³⁵⁾ to assess the time period when orb2 function is required. This system uses ubiquitous expression of GAL80^{ts} to conditionally suppress a GAL4-driven transgene; at 18 °C, GAL80^{ts} shuts off GAL4 activity, but at 29 °C GAL80^{ts} is inactive and GAL4 is active. We used this system with 247-GAL4 and either UAS-orb2A or UAS-orb2B to conditionally express either of the Orb2 isoforms in mushroom bodies (Fig. 5a). In the initial control experiments, we found that mushroom body expression of either UAS-orb2A or UAS-orb2B fully rescued long-term memory in orb2^{ΔQ}/orb2^Δ mutants when these flies were raised and tested at 29 °C (Fig. 5b and Supplementary Table 8 online, Experiment 1), but not when they were raised and tested at 18 °C (Supplementary Table 8, Experiment 2). Long-term memory was also restored when flies were raised at 18 °C and then shifted to 29 °C immediately after eclosion, thereby providing orb2 function only in adults (Supplementary Table 8, Experiment 3). The reciprocal experiment, in which flies were raised at 29 °C, but shifted to 18 °C as adults, did not restore long-term memory (Supplementary Table 8, Experiment 4). These data establish that orb2 function is required in the adult mushroom bodies for long-term memory.

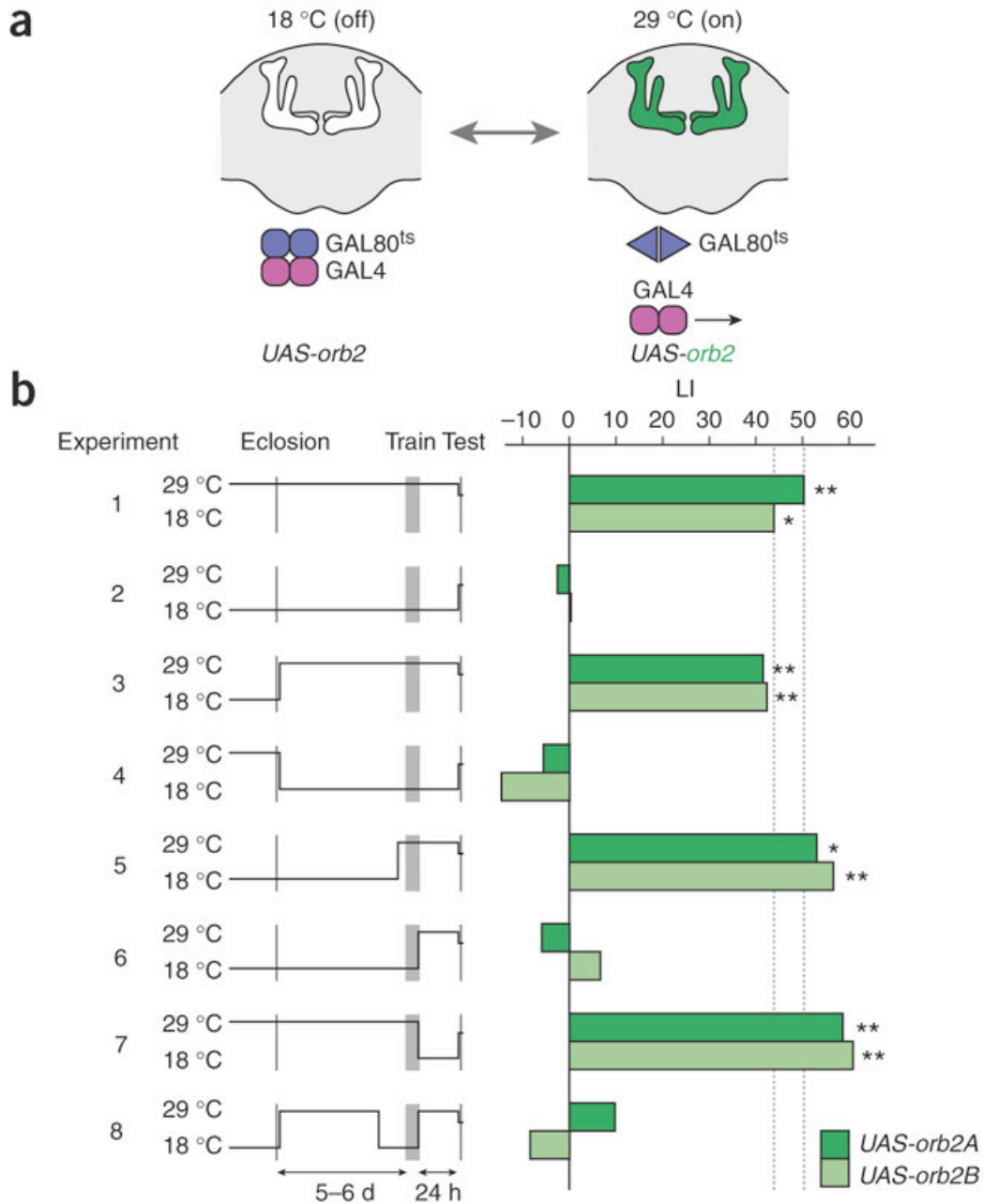


Figure 5: orb2 function is required during or shortly after training.

(a) Schematic showing how the TARGET system was used to conditionally express an orb2 transgene in the mushroom bodies. (b) LIs of 247-GAL4, orb2^Δ/UAS-orb2A, tub-GAL80^{ts}, orb2^{ΔQ} males (right) cultured according to eight different temperature regimes (left) in tests for memory of courtship conditioning carried out 24 h after a 5-h training period (Supplementary Table 8). Where indicated, temperature shifts were applied immediately after eclosion, 2 or 24 h before training or immediately after training. In all cases, males were shifted to 25 °C 1 h before testing. *P < 0.002, **P < 0.0001 for H₀, LI = 0 (permutation test).

To further dissect the temporal requirements for orb2 function, we carried out an additional series of TARGET experiments in which the temperature was shifted either just before or just after training (Fig. 5b and Supplementary Table 8, Experiments 5–8). We found that flies that were shifted up to 29°C just 2 h before training showed normal long-term memory (Experiment 5), whereas those that were shifted immediately after training had no long-term memory (Supplementary Table 8, Experiment 6). Conversely, flies shifted down to 18°C immediately after training had normal long-term memory (Supplementary Table 8, Experiment 7), whereas flies shifted down to 18°C 24 h before training, but then shifted up to 29°C immediately after training, did not (Supplementary Table 8, Experiment 8). We do not know the precise dynamics of Orb2 regulation using this system, but the last experiment indicates that 24 h at 18°C is sufficient to downregulate orb2 function. If the dynamics of Orb2 turnover are the same after training as they are before (which is not necessarily the case), then these last two experiments further suggest that orb2 function is not required during testing. We infer from these data that long-term memory critically requires Orb2 activity in the mushroom body neurons during or shortly after training.

2.2.6 orb2 function is required in mushroom body γ neurons

Finally, we sought to further refine the cellular requirement for orb2 function in long-term courtship memory. For this, we expressed the UAS-orb2A transgene with a set of GAL4 drivers specific for different subsets of mushroom body neurons (Fig. 6a–e). We also tested a driver for olfactory projection neurons, the major source of input to the mushroom bodies (Fig. 6f). Expression of orb2 with some of these drivers resulted in developmental lethality, presumably as a result of additional expression outside of the mushroom bodies. To circumvent this problem, we used the TARGET system with all of these drivers to restrict expression of the UAS-orb2A transgene to adults (as in Fig. 5b, Experiment 1).

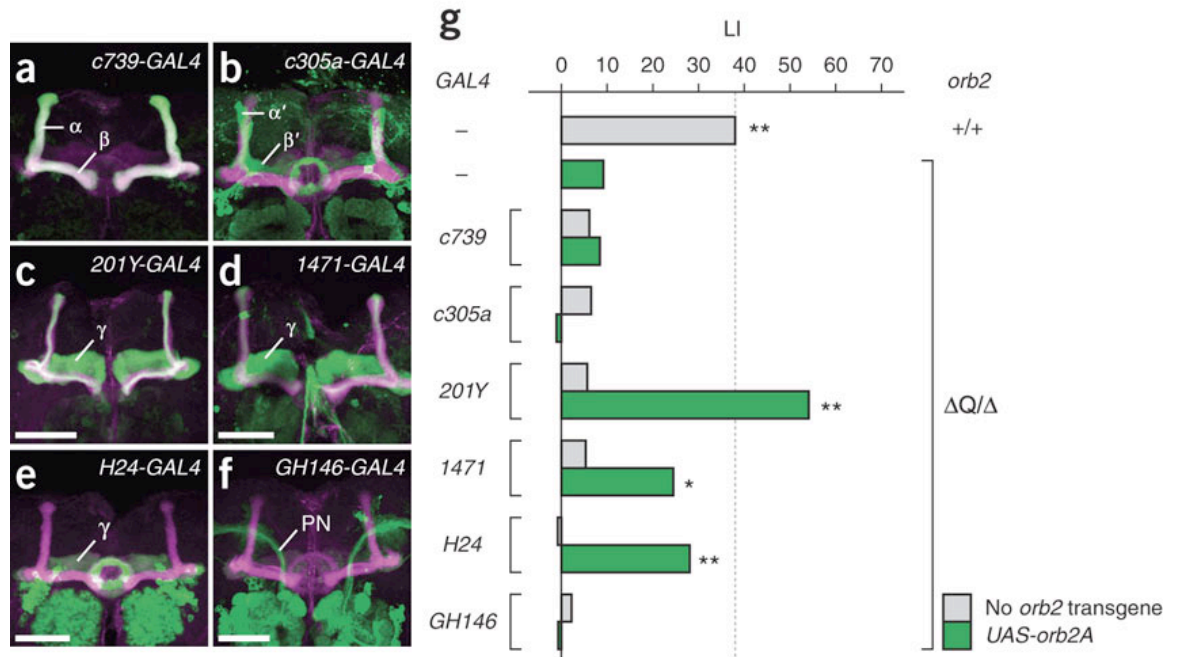


Figure 6: orb2 is required in mushroom body γ neurons.

(a–f) Confocal projections through the mushroom body lobes of adults carrying the indicated GAL4 drivers together with UAS-CD8-GFP, stained with antibody to GFP (green) and counterstained with antibody to FasII (magenta) to visualize the $\alpha\beta$ and γ neurons. Scale bars, 50 μ m. (g) LI of wild-type (+/+) or orb2 ^{ΔQ} /orb2 ^{Δ} males in tests of courtship conditioning carried out 24 h after a 5-h training period (Supplementary Table 9). GAL4 drivers were used to express UAS-orb2A in the orb2 mutant background as indicated (– denotes no GAL4 driver), using the TARGET system to restrict transgene expression to adults. Note that 201Y-GAL4 and 1471-GAL4 drive expression not only in γ neurons, but also in a small subset of $\alpha\beta$ neurons. Because the $\alpha\beta$ driver c739-GAL4 did not provide rescue, but the γ drivers fruGAL4 and H24-GAL4 did, we infer that rescue with 201Y-GAL4 and 1471-GAL4 is a result of expression in γ neurons. *P < 0.001, **P < 0.0001 for H₀, LI = 0 (permutation test).

When we expressed orb2A in the mushroom body $\alpha\beta$ neurons with the c739-GAL4 driver³⁶, in the $\alpha'\beta'$ neurons with c305a-GAL4 (ref. 37) or in projection neurons with GH146-GAL4 (ref. 38), we observed no rescue of long-term memory (Fig. 6g and Supplementary Table 9 online). In contrast, long-term memory was rescued when we expressed orb2A with 201Y-GAL4 (ref. 33), 1471-GAL4 (ref. 34) or H24-GAL4 (ref. 33), each an independent driver for the mushroom body γ neurons, (Fig. 6g and Supplementary Table 9). We also

observed that fruGAL4 was primarily expressed in the mushroom body γ neurons⁽²⁰⁾ (Fig. 4a). The simplest interpretation of these data is that long-term memory of courtship conditioning requires Orb2 function specifically in fru-positive mushroom body γ neurons.

2.3 Discussion

On the basis of their synaptic localization and their ability to regulate mRNA translation, CPEB proteins have been proposed to act as key regulators of the local protein synthesis that underlies long-term memory formation^(12, 13, 14, 16, 17). Behavioral evidence to support this view has been lacking to date. Here, we have demonstrated that a CPEB protein is indeed required for a long-term behavioral memory. Specifically, we have shown that *Drosophila* Orb2 is essential for long-term courtship memory. We have mapped this requirement spatially to fru-positive mushroom body γ neurons and temporally to the period during or shortly after training. Whether Orb2 acts pre- or postsynaptically in these neurons remains an open question, as does the identity and function of the mRNAs that it might regulate.

Courtship conditioning is thought to be a form of associative learning and most likely involves modulation of the male's response to female pheromones^(39, 40). We chose this learning and memory assay because of both its robustness and our increasing understanding of the innate template for courtship behavior^(19, 20, 41). Male courtship behavior is guided, at least in part, by volatile pheromones that activate the three sexually dimorphic fru-positive glomeruli in the antennal lobe^(20, 42, 43). Some of the projection neurons that innervate these glomeruli are likely to connect directly to mushroom body γ neurons⁽⁴⁴⁾, which in turn have been shown to require fru function for short-term courtship conditioning⁽¹⁹⁾. The fru-positive mushroom body γ neurons thus emerge as critical plasticity elements in the neural circuitry that is involved in pheromone processing. We envision that orb2 may be required in other mushroom body neurons for other forms of long-term olfactory memory, and in other regions of the nervous system for diverse

forms of long-term plasticity. It will be important to test these predictions using other learning and memory procedures.

One appealing model for long-term memory formation proposes that CPEB proteins switch into an active prion-like conformation, forming a long-lasting molecular mark at activated synapses⁽¹⁷⁾. We deleted the glutamine-rich region of *Drosophila* Orb2 in part to explore this idea, as it is the corresponding region of *Aplysia* CPEB that mediates prion formation in vitro⁽¹⁷⁾. Consistent with the prion hypothesis, we found that these mutants were impaired in long-term memory formation. Moreover, these mutants did not appear to disrupt other Orb2 functions, arguing against alternative models in which the glutamine-rich region has a more general role in Orb2 function. This mirrors the observation that the prion domains of yeast proteins are similarly dispensable for their normal biochemical activities⁽⁴⁵⁾. However, our data also do not exclude much simpler models of Orb2 regulation. For example, the glutamine-rich region might be an interaction site for factors that regulate Orb2 in response to synaptic activity, analogous to the manner in which *Xenopus* CPEB is activated during oocyte maturation⁽⁴⁶⁾.

Regardless of the exact mechanism, our behavioral studies have demonstrated that Orb2 is required for long-term memory, but is dispensable for learning, short-term memory and memory recall. This is precisely the phenotype that is predicted after the loss of the synaptic tag that is postulated to mark specific synapses for the protein synthesis–dependent changes that underlie long-term behavioral memory. The molecular features of Orb2 suggest that it could be either a component or a regulator of this tag.

2.4 Supplementary information

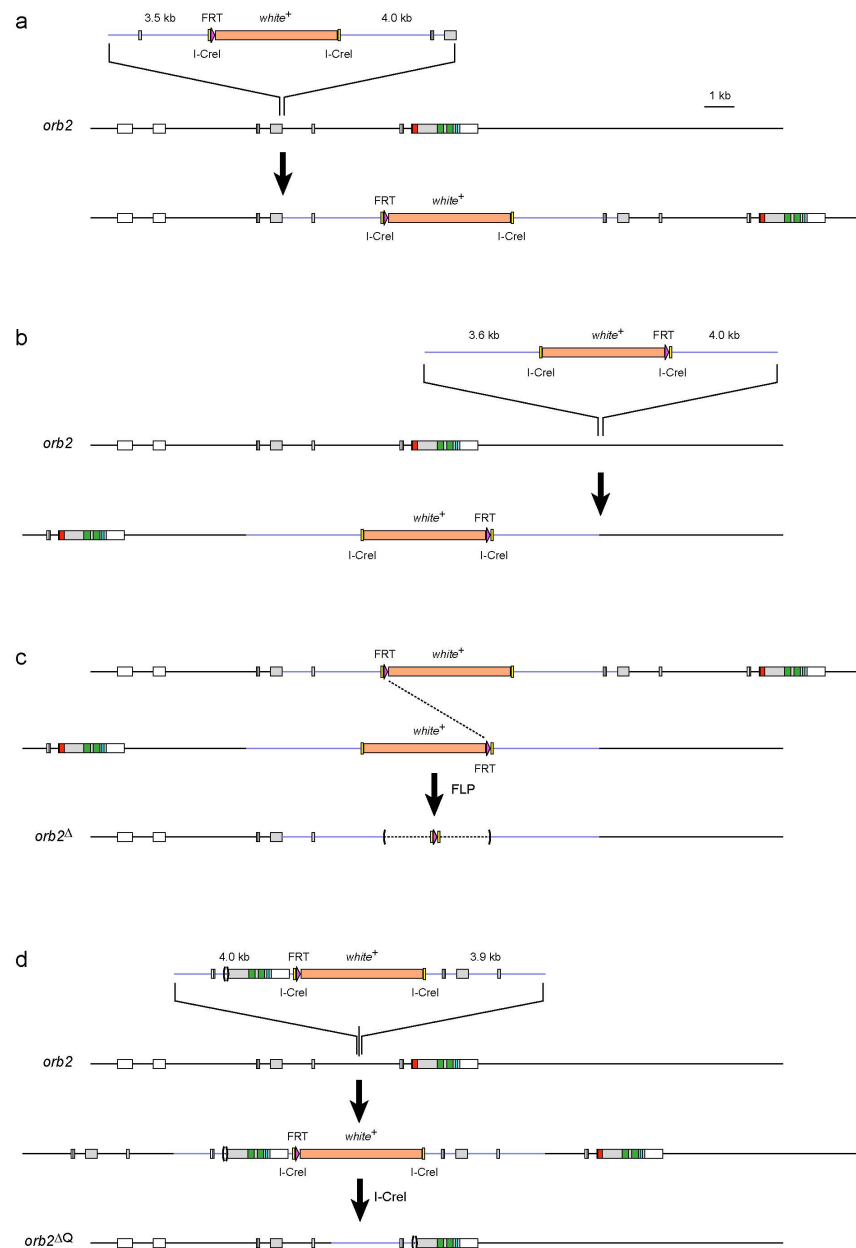
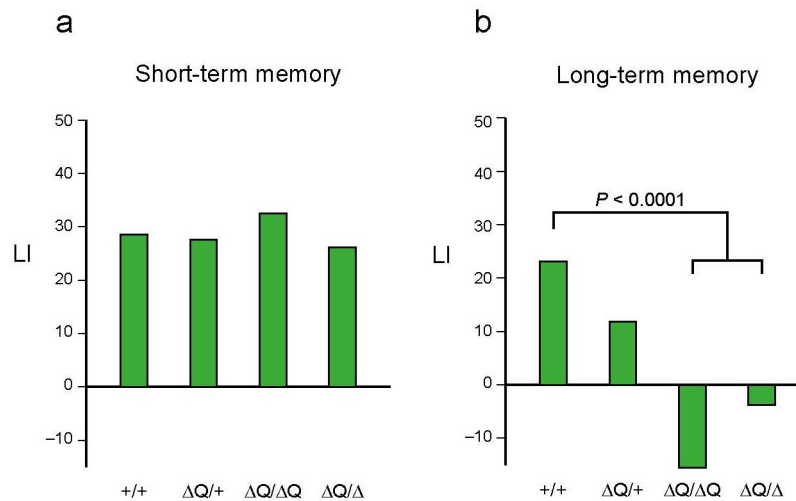


Figure S1

Supplementary Fig. 1 *orb2* targeting strategy.

(a-c) Targeting strategy used to generate *orb2*^Δ. An FRT site was inserted 5' to the first exon of the RA transcript (a), and 3' to the common last exon (b). Targeting

constructs are shown in their linear form (following FLP-induced excision from the original P-element donor transgene, and linearization by I-SceI), with homology arms shown in blue. Ends-in targeting was used, thereby generating a duplication that includes the *white*⁺ selection marker and flanking FRT and I-CreI sites. FLP-induced recombination at FRT sites generated the final *orb2* Δ allele, selected by the loss of the *white*⁺ marker (**c**). The I-CreI sites were not used in this strategy. (**d**) Targeting strategy used to generate *orb2* Δ Q. The donor construct carries the Δ Q mutation in one of its homology arms, generated by overlap extension PCR. The duplication generated by ends-in targeting was resolved in this case by using I-CreI to generate a doublestranded break and selecting for loss of the *white*⁺ marker in the progeny. PCR was then used to select lines that carried the Δ Q mutation, but no intact copy of this exon.



Supplementary Fig. 2 Memory assays with anesthetized virgins.

Learning indices of males carrying the indicated *orb2* alleles, trained with mated females and tested with an anesthetized virgin females. (a). Short-term memory test 30 min after a 1.5-h training period (**Supplementary Table 1** online). (b). Long-term memory test 24 h after a 5-h training period (**Supplementary Table 3** online). *P* values are for $H_0: LI = LI_{+/+}$ (permutation test).

Supplementary Table 1. Short-term memory tested with anesthetized virgin

Genotype	Naïve		Trained		LI	<i>P-values</i>	
	CI	<i>n</i>	CI	<i>n</i>		LI=0	LI=LI _{+/+}
+/+	87.9±6.1	24	62.9±6.3	27	28.5	0.0032	
ΔQ/+	93.6±2.1	24	67.7±6.4	27	27.6	0.0001	0.93
ΔQ/ΔQ	76.4±5.3	42	51.5±5.0	46	32.5	0.0006	0.74
ΔQ/Δ	95.9±1.6	38	70.8±5.1	46	26.2	<0.0001	0.77

Courtship conditioning data for males of the indicated genotypes tested with an anaesthetized virgin female 30 min after a 1.5-h training session. Data for CI are mean ± s.e.m.

Supplementary Table 2. Short-term memory tested with mated female

Genotype	Naïve		Trained		LI	<i>P-values</i>	
	CI	<i>n</i>	CI	<i>n</i>		LI=0	LI=LI _{+/+}
+/+	79.2±5.1	38	60.9±6.1	42	23.1	0.012	
ΔQ/+	85.2±4.1	28	75.1±5.8	28	11.8	0.081	0.21
ΔQ/ΔQ	70.2±5.5	42	81.0±4.0	37	-15.5	0.94	0.0001
ΔQ/Δ	87.6±5.3	26	90.9±2.7	30	-3.8	0.71	<0.0001

Courtship conditioning data for males of the indicated genotypes tested with a mated female 30 min after a 1.5-h training session. Data for CI are mean ± s.e.m.

Supplementary Table 3. Long-term memory tested with anesthetized virgin

Genotype	Naïve		Trained		LI	<i>P-values</i>	
	CI	<i>n</i>	CI	<i>n</i>		LI=0	LI=LI _{+/+}
+/+	72.0±6.0	20	23.5±2.8	31	67.3	<0.0001	
ΔQ/+	80.8±4.8	24	22.7±4.6	40	71.9	<0.0001	0.84
ΔQ/ΔQ	88.0±4.0	33	29.1±4.4	43	66.9	<0.0001	0.98
ΔQ/Δ	91.2±1.6	69	33.4±3.7	78	63.4	<0.0001	0.71

Courtship conditioning data for males of the indicated genotypes tested with an anaesthetized virgin female 24 h after a 5-h training session. Data for CI are mean ± s.e.m.

Supplementary Table 4. Long-term memory tested with mated female

Genotype	Naïve		Trained		LI	<i>P-values</i>	
	CI	<i>n</i>	CI	<i>n</i>		LI=0	LI=LI _{+/+}
+/+	69.3±4.5	41	32.2±4.7	43	53.6	<0.0001	
ΔQ/+	84.4±3.0	60	58.8±4.4	61	30.3	<0.0001	0.0056
ΔQ/ΔQ	80.2±2.9	68	80.8±2.4	74	-0.7	0.56	<0.0001
ΔQ/Δ	89.3±2.1	70	84.5±2.4	68	5.4	0.072	<0.0001

Courtship conditioning data for males of the indicated genotypes tested with a mated female 24 h after a 5-h training session. Data for CI are mean ± s.e.m.

Supplementary Table 5. Memory decay

Training period	Time until testing	Naïve		Trained		LI	P-values	
		CI	<i>n</i>	CI	<i>n</i>		LI=0	LI=LI _{0.5}
+/+								
5 h	0.5 h	73.4±6.2	12	23.3±4.9	24	68.3	<0.0001	
5 h	6 h	71.0±4.0	38	27.6±3.9	49	61.1	<0.0001	0.65
5 h	12 h	79.8±4.2	23	21.7±4.2	29	72.8	<0.0001	0.84
5 h	24 h	85.4±2.3	43	37.4±4.4	44	56.3	<0.0001	0.31
ΔQ/Δ								
5 h	0.5 h	86.0±3.9	36	21.6±4.6	39	74.9	<0.0001	
5 h	3 h	92.6±4.2	8	25.8±8.7	10	72.1	<0.0001	0.94
5 h	4 h	92.8±3.1	8	37.2±8.6	10	59.9	<0.0001	0.58
5 h	5 h	93.1±1.8	8	25.8±8.9	11	72.3	<0.0001	0.95
5 h	6 h	81.9±4.9	25	29.5±5.1	36	64.0	<0.0001	0.58
5 h	7 h	81.2±5.1	12	39.4±6.7	17	51.4	0.0001	0.26
5 h	8 h	88.5±7.5	4	56.8±11.6	6	35.8	0.031	0.14
5 h	9 h	91.5±1.3	12	78.7±5.9	14	14.0	0.023	<0.0001
5 h	12 h	93.1±5.3	11	97.0±1.1	18	-4.2	0.68	<0.0001
5 h	18 h	94.8±1.7	12	85.0±8.0	12	10.4	0.12	<0.0001
5 h	24 h	85.6±4.9	19	77.5±7.7	18	9.4	0.19	<0.0001

Courtship conditioning data for males of the indicated genotypes tested with a mated female at various intervals after a 5-h training session. Data for CI are mean ± s.e.m

Supplementary Table 6. Effect of training duration

Training period	Time until testing	Trained		LI	<i>P-values</i>	
		CI	<i>n</i>		LI=0	LI=LIsh
+/+						
naïve	6 h	52.2±5.8	12			
1 h	6 h	31.3±5.8	12	39.9	0.0095	0.45
5 h	6 h	19.7±5.8	12	62.3	0.0003	
8 h	6 h	16.2±4.4	12	69.0	<0.0001	0.83
naïve	8 h	41.4±5.3	12			
1 h	8 h	48.0±8.6	12	-15.8	0.74	0.026
5 h	8 h	17.1±3.9	12	58.8	0.0008	
8 h	8 h	17.0±2.7	11	58.9	0.0004	1.0
naïve	24 h	62.8±6.0	12			
1 h	24 h	64.5±7.4	12	-2.7	0.57	0.052
5 h	24 h	37.4±7.3	12	40.5	0.0078	
8 h	24 h	28.6±8.0	12	54.5	0.0018	0.60
ΔQ/Δ						
naïve	6 h	65.4±6.3	12			
1 h	6 h	42.4±7.5	12	35.3	0.015	0.29
5 h	6 h	22.2±6.6	12	66.1	0.0002	
8 h	6 h	29.5±5.7	12	54.9	0.0001	0.64
naïve	8 h	64.9±7.6	12			
1 h	8 h	61.0±5.8	12	6.1	0.34	0.82
5 h	8 h	58.1±8.9	12	10.5	0.28	
8 h	8 h	68.0±9.3	12	-4.7	0.60	0.40
naïve	12 h	80.7±5.8	10			
1 h	12 h	84.8±3.8	10	-5.1	0.71	0.39
5 h	12 h	76.3±7.1	12	5.4	0.33	
8 h	12 h	76.8±6.3	12	4.9	0.33	0.97
naïve	24 h	90.2±4.4	12			
1 h	24 h	87.8±7.4	9	2.6	0.38	0.30
5 h	24 h	78.2±6.5	12	13.3	0.071	
8 h	24 h	90.1±5.6	12	0.1	0.49	0.070

Courtship conditioning data for wild-type and *orb2* mutant males tested with a mated female at various intervals after a 1-h, 5-h, 8-h or sham training session. Data for CI are mean ± s.e.m.

Supplementary Table 7. Transgenic rescue of long-term memory

Genotype			Naïve		Trained			P-values	
<i>orb2</i>	<i>GAL4</i>	<i>UAS-orb2</i>	CI	<i>n</i>	CI	<i>n</i>	LI	LI=0	LI=LI+/-
+/+	-	-	70.8±3.5	34	34.1±4.9	23	51.9	<0.0001	
ΔQ/Δ	-	A	78.5±3.4	47	83.6±2.5	56	-6.4	0.89	<0.0001
ΔQ/Δ	-	B	74.8±4.0	46	77.4±3.5	51	-3.5	0.69	<0.0001
ΔQ/Δ	<i>fru</i>	-	60.1±5.5	36	67.8±4.2	36	-12.7	0.87	<0.0001
ΔQ/Δ	<i>fru</i>	A	43.7±6.1	24	12.0±3.5	22	72.6	<0.0001	0.49
ΔQ/Δ	<i>fru</i>	B	68.3±3.8	47	36.3±4.6	47	46.8	<0.0001	0.70
ΔQ/Δ	<i>c772</i>	-	74.9±7.3	20	74.4±6.1	21	0.6	0.48	0.0003
ΔQ/Δ	<i>c772</i>	A	69.1±5.4	35	35.0±6.1	36	49.3	0.0001	0.88
ΔQ/Δ	<i>c772</i>	B	69.3±6.0	35	28.6±5.6	36	58.8	<0.0001	0.72
ΔQ/Δ	247	-	75.4±4.0	36	70.2±4.7	34	6.9	0.20	<0.0001
ΔQ/Δ	247	A	86.5±3.3	36	49.5±5.3	43	42.7	<0.0001	0.43
ΔQ/Δ	247	B	85.4±2.7	45	49.1±4.5	56	42.6	<0.0001	0.36

Courtship conditioning data for males of the indicated genotypes tested with a mated female 24 h after a 5-h training session. Data for CI are mean ± s.e.m.

Supplementary Table 8. TARGET experiments

experiment	<i>UAS-orb2</i>	Naïve		Trained			P-values	
		CI	<i>n</i>	CI	<i>n</i>	LI	LI=0	LI=LI1
1	A	71.7±5.3	27	35.6±5.7	38	50.3	<0.0001	
1	B	69.8±6.1	25	39.2±6.8	27	43.9	0.0011	
2	A	69.8±5.5	33	71.7±5.0	40	-2.7	0.60	<0.0001
2	B	65.8±5.5	36	65.5±4.4	51	0.4	0.49	0.0004
3	A	71.9±4.2	30	42.1±5.1	39	41.5	<0.0001	0.53
3	B	74.9±4.3	34	43.2±4.7	36	42.4	<0.0001	0.90
4	A	69.2±6.4	27	73.1±4.6	37	-5.6	0.70	<0.0001
4	B	65.0±7.1	26	74.6±5.6	31	-14.7	0.86	0.0002
5	A	68.5±8.2	24	32.2±7.8	20	53.1	0.0002	0.89
5	B	74.3±4.2	26	32.3±5.6	27	56.6	<0.0001	0.46
6	A	63.4±5.9	32	67.7±4.2	57	-6.0	0.71	0.0001
6	B	66.5±5.9	31	62.1±5.7	36	6.7	0.30	0.0074
7	A	65.7±5.6	24	27.2±3.7	37	58.6	<0.0001	0.66
7	B	64.0±5.7	27	25.1±4.0	36	60.8	<0.0001	0.40
8	A	86.2±3.9	24	77.6±3.6	49	10.0	0.060	<0.0001
8	B	80.2±3.9	20	87.0±3.3	28	-8.5	0.90	<0.0001

Courtship conditioning data for $247\text{-}GAL4$, $orb2^{\Delta} / UAS\text{-}orb2A$, $tub\text{-}GAL80^{ts}$, $orb2^{\Delta Q}$ males raised, trained and tested with mated females according to the regimes indicated in **Fig. 6b**. Data for CI are mean $\pm$ s.e.m.

Supplementary Table 9. Mapping the *orb2* requirement to specific mushroom body subsets

Genotype			Naïve		Trained			P-values	
<i>orb2</i>	<i>GAL4</i>	<i>UAS-orb2</i>	CI	<i>n</i>	CI	<i>n</i>	LI	LI=0	LI=LI+/-
+/+	-	-	75.0 $\pm$ 4.8	36	46.5 $\pm$ 5.4	42	38.0	0.0001	
$\Delta Q/\Delta$	-	A	90.8 $\pm$ 3.9	29	82.5 $\pm$ 3.2	60	9.2	0.06	0.0001
$\Delta Q/\Delta$	<i>c739</i>	-	90.9 $\pm$ 3.5	38	85.3 $\pm$ 4.1	41	6.1	0.15	<0.0001
$\Delta Q/\Delta$	<i>c739</i>	A	78.1 $\pm$ 3.9	53	71.5 $\pm$ 4.2	59	8.4	0.13	0.0004
$\Delta Q/\Delta$	<i>c305a</i>	-	91.5 $\pm$ 2.8	35	85.5 $\pm$ 3.9	41	6.5	0.12	<0.0001
$\Delta Q/\Delta$	<i>c305a</i>	A	73.7 $\pm$ 5.5	30	74.5 $\pm$ 4.6	44	-1.1	0.55	0.0007
$\Delta Q/\Delta$	<i>201Y</i>	-	90.6 $\pm$ 3.3	44	85.4 $\pm$ 4.0	45	5.7	0.17	<0.0001
$\Delta Q/\Delta$	<i>201Y</i>	A	79.0 $\pm$ 4.0	55	36.3 $\pm$ 4.7	56	54.1	<0.0001	0.21
$\Delta Q/\Delta$	<i>1471</i>	-	89.3 $\pm$ 4.3	19	84.6 $\pm$ 4.1	28	5.3	0.43	<0.0001
$\Delta Q/\Delta$	<i>1471</i>	A	76.9 $\pm$ 3.8	48	58.1 $\pm$ 4.3	51	24.5	0.0009	0.14
$\Delta Q/\Delta$	<i>H24</i>	-	80.9 $\pm$ 4.6	37	81.6 $\pm$ 4.0	42	-0.8	0.55	<0.0001
$\Delta Q/\Delta$	<i>H24</i>	A	82.7 $\pm$ 3.5	56	59.5 $\pm$ 4.9	56	28.1	0.0001	0.27
$\Delta Q/\Delta$	<i>GH146</i>	-	91.2 $\pm$ 3.6	40	89.2 $\pm$ 4.1	40	2.2	0.36	<0.0001
$\Delta Q/\Delta$	<i>GH146</i>	A	86.5 $\pm$ 3.5	36	87.2 $\pm$ 3.7	34	-0.7	0.54	<0.0001

Courtship conditioning data for males of the indicated genotypes tested with a mated female 24 h after a 5-h training session. *orb2* mutants additionally carried a *tubGAL80^{ts}* transgene. For all genotypes, males were raised at 18°C and shifted to 29°C shortly after eclosion. Data for CI are mean $\pm$ s.e.m

2.5 Methods

Bioinformatic analysis.

The phylogenetic tree (Fig. 1a) was prepared using the neighbor-joining algorithm with support estimated by 1,000 bootstrap replicates. NCBI protein sequence database accession numbers: AAK52834, NP_524460, NP_001017330, NP_031781, NP_729429, NP_080528, NP_787951, NP_001015925 and NP_938042. *Xenopus tropicalis* CPEB2 and CPEB4 sequences were derived from genomic and expressed sequence tag information.

In situ hybridization.

RNA probes for in situ hybridization were prepared from a pBluescript SK+ clone containing a 657–base pair fragment from the exon that is common to all predicted orb2 transcripts (Fig. 1b). The antisense probe was prepared using T7 polymerase and the sense probe was prepared with T3 polymerase. Probe synthesis was confirmed by gel electrophoresis. Probes were hybridized overnight at 60 °C to 10- μ m cryosections of whole flies or fly heads embedded in Tissue-Tek OCT compound, essentially as previously described⁽⁴⁷⁾. After washing, slides were stained overnight at 4 °C with mAbdac2-3 (1:200, Developmental Studies Hybridoma Bank) and alkaline phosphatase–conjugated antibody to digoxigenin Fab fragments (1:2,000, Roche). For fluorescent detection, the slides were incubated for 1 h with Alexa Fluor 488 goat antibody to rabbit IgG (1:500, Molecular Probes) and were subsequently incubated with Fast Red (Dako) for 3 h at 20–22°C. Slides were mounted in Vectashield DAPI medium (Vector Labs), and confocal images were

acquired on a Zeiss LSM 510 microscope and further processed in Adobe Photoshop using linear brightness and contrast adjustments.

Generation and verification of orb2 mutants.

Gene targeting by homologous recombination⁽⁴⁸⁾ was carried out according to the strategy outlined in Supplementary Figure 1, using donor constructs located on the second chromosome. These donor constructs included homology regions prepared by PCR amplification from genomic DNA isolated from Canton S flies. Following either FLP-mediated recombination (orb2) or I-CreI-mediated excision (orb2Q), the intended modifications were verified by PCR amplification and DNA sequencing across the deleted region. For RT-PCR, fly heads were collected by freezing in liquid nitrogen and passing them through a metal sieve. Total RNA was extracted using Trizol, reverse transcribed with random primers and amplified by PCR using primer B (5'-GAT GAA GAA CAA CGG GCT GGG ATT-3') and primer C (5'-GGG GAG GAA TGG GCG AGA TG-3'). Behavioral assays.

Flies were raised on semidefined medium⁽⁴⁹⁾ at 25 °C (unless otherwise indicated) and 60–70% humidity in a 12-h light-dark cycle. Males and females were collected at eclosion; males were aged for 5–6 d in isolation and females were aged for 3–5 d in groups of 50–100. Canton S females were premated by pairing 10 females with 15 Canton S males (>3 d old) in food vials for 18 h, and then recovered 2–3 h before being used for training. Males were assayed for courtship conditioning using the repeat training test^(24, 50). For training, individual males were placed in food chambers either with (trained) or without (naive) a single premated female. After training (or sham training), each male was recovered, transferred to a fresh food vial and kept in isolation at the appropriate temperature until testing. For the TARGET experiments, males were transferred to 25 °C 1 h before testing. All tests were carried out by pairing each male with a

fresh predated female in a 1-cm courtship chamber for 10 min. Tests were videotaped, and all 5,436 assays were manually scored for the courtship index, blind to the genotype and, as far as possible, the experimental condition. Statistical analyses were carried out using a custom MATLAB (Mathworks) script to perform permutation tests⁽²⁴⁾. Briefly, the entire set of courtship indices for both the naive and trained flies were pooled and then randomly assorted into simulated naive and trained sets of the same size as in the original data. A LI_p was calculated for each of 100,000 randomly permuted datasets, and P values were estimated as the fraction for which $LI_p > LI$ (to test $H_0, LI = 0$) or $|LI_p| > |LI - LI_0|$ (to test $H_0, LI = LI_0$).

Rescue experiments.

UAS-orb2A and UAS-orb2B were prepared by PCR amplification of the entire orb2 coding region and 3' untranslated regions from the EST clones AT07721 and RE26128, respectively, and inserting these fragments into pUAST. A third chromosome insertion of each transgene was recombined onto the orb2Q chromosome. For TARGET experiments, a tub-GAL80^{ts} transgene on the third chromosome⁽³⁵⁾ was additionally recombined onto this chromosome. For the TARGET experiments presented in Fig. 5, the indicated temperature shifts were applied; for those presented in Figure 6, flies were raised at 18 °C and then shifted to 29 °C shortly after eclosion.

Author contributions

B.J.D. and K.K. designed the experiments. K.K. generated the orb2 alleles and carried out all anatomical and behavioral studies together with S.K. M.A. performed the in situ hybridization experiments. B.J.D. wrote the manuscript.

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3. Molecular mechanisms of Orb2 function in long-term memory

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3.1 Introduction

Long-term memory formation is thought to reflect experience dependent modifications at specific synapses. These are, at least in part, mediated by local translation of mRNAs to strengthen existing synapses and to build up new connections. Because mRNAs are in theory available to all synapses of a neuron (Klann and Dever, 2004; Sutton and Schuman, 2006), it has been speculated that some molecules present in the synapse might tightly control mRNA translation to ensure translation happens only at the appropriate synapses (Martin et al., 1997).

Cytoplasmic Polyadenylation Element binding Proteins (CPEBs) are ideal candidates to mediate such a local translation. Some members of the CPEB1 subfamily have been shown to localize to synapses (Si et al., 2003a; Wu et al., 1998) and contribute to long-term depression (Alarcon et al., 2004) and facilitation (Si et al., 2003a). These proteins control translation by cytoplasmic polyadenylation and bind to short canonical sequences called cytoplasmic polyadenylation elements (CPE elements). The CPE elements have a consensus sequence $U_{4-5}A_{1-3}U$ that resides in the 3'UTRs of their target mRNAs (Fox et al., 1989). When CPEBs are bound to the CPEs they stabilize the mRNA by polyadenylation (Fox et al., 1989; Hake and Richter, 1994). However, while AcCPEB1 has been shown to stabilize long-term facilitation beyond 48hrs (Miniaci et al., 2008), behavioral tests on CPEB1 knockout mice did not show the

expected phenotype in long-term memory formation, but showed only a role in memory extinction (Berger-Sweeney et al., 2006).

Interestingly, members of a second, more recently identified class of CPEB proteins (hereafter called CPEB2-4 subfamily) (Theis et al., 2003), with the vertebrate mCPEB2, 3 and 4, are also expressed in the brain, where they localize to post-synaptic densities (Huang et al., 2006). Members of this subfamily are also involved in control of the mRNA translation by mechanisms that might involve neither polyadenylation nor binding of CPE elements (Huang et al., 2006). Currently, no mouse knock-out of mCPEB2-4 family members has been published, therefore the role for mCPEB2-4 in long-term memory formation is still subject to debate.

In order to assess the relative contributions of CPEB2-4 family to memory formation, our lab has previously looked at genetic perturbations of the *Drosophila* homologue Orb2 (Keleman et al., 2007). We have shown that Orb2 is acutely required for long-term memory but is dispensable for short-term memory (Keleman et al., 2007). The temporal requirement was mapped to the time of training but was shown to be dispensable for retrieval of memory. However, the molecular mechanism of Orb2 function in memory formation was not yet described.

Orb2, like all other members of its subfamily, lacks a consensus sequence for Aurora A phosphorylation (Liu and Schwartz, 2003; Si et al., 2003a), required for activation of the CPEB1 subfamily (Mendez et al., 2000). As an alternative model for regulation it has been suggested that a conformational change of CPEB proteins, which involves self-association to a prion-like aggregate could have a similar function. Surprisingly, it has been shown that AcCPEB1 in aggregated form is able to regulate mRNA translation (Si et al., 2010; Si et al., 2003a; Si et al., 2003b). This aggregation property might also explain the long lasting nature of long-term memory in contrast to the relatively fast protein turnover in synapses. Similar to AcCPEB1, also Orb2 has been suggested to form such an aggregated state, at least when over expressed in cultured neurons (Si et al., 2010). Both proteins carry a low complexity region in their N-terminus,

which is highly enriched in glutamine thought to mediate the self-association. To date the best evidence that this domain might have a functional role in long-term memory formation comes from deletion mutants. Experiments deleting specifically the Q-enriched, and putative prion forming domain, leads to a disturbed long-term memory but leaves short-term memory intact (Keleman et al., 2007). However, experiments elucidating the exact role of this domain have not been reported yet.

Here we address the question of the mechanism behind Orb2 function in long-term memory formation. We substituted most of the orb2 locus with an attP-site so that we can readily modify its endogenous genetic locus. This has allowed us to do a large-scale structure/function study of the orb2 gene while maintaining endogenous expression levels.

Using this system we tagged orb2 with various epitope tags and found it to be highly enriched in the nervous system throughout all developmental stages and to be present in synapses in both pre- and post-synaptic compartments. Importantly, these tagged orb2 constructs fully rescue orb2 mutants.

Orb2 encodes two different isoforms that differ in their N-terminus. We have established that the Orb2A isoform is not required during development of the nervous system but is exclusively required for long-term memory, while the Orb2B isoform seems to function in both. The function of the Orb2A isoform is critically dependent on the N-terminal glutamine stretch. Homologous Q-rich domains of other CPEB proteins can functionally replace the Orb2 Q-domain and restore normal long-term memory. This suggests that the specific sequence of the putative prion domain is not important, rather it is the presence of this domain that is vital for Orb2 function in long-term memory. When over-expressed in S2 cells only Orb2A but not Orb2B was found to form punctuate structures. Importantly these aggregates disappeared upon removal of the glutamine domain.

Unlike the N-terminal glutamine rich domain, the RNA binding domain (RBD) of Orb2 cannot be functionally replaced with other CPEB family members. Chimeric orb2 constructs with CPEB1 family RNA binding domains provide no

rescue. By contrast, orb2 constructs with CPEB2 family RNA binding domains provide some rescue of the developmental defects but essentially no rescue of long-term memory. Hence, the underlying RNA targets are likely to be more conserved with respect to developmental functions than they are for the long-term memory formation.

Point mutations, predicted to interfere with the function of the RNA binding domain lead to lethality. However, this domain seems to be only critically required in Orb2B but to be dispensable in Orb2A. This poses the question of how Orb2 mediates long-term memory.

3.2 Results

3.2.1 The Orb2^{attP} allele allows structure/function analysis of the Orb2 gene

Previous experiments on Orb2 (Keleman et al., 2007) demonstrated a role in long-term memory but did not address the molecular mechanism for Orb2 function in long-term memory formation. In order to get at the underlying molecular mechanism, we decided to take a structure/function approach where each domain of Orb2 can be independently analysed through targeted modification of the endogenous locus. Targeted modification of orb2 locus allows expression of modified Orb2 proteins in the precise expression pattern of endogenous Orb2, and thus specific functions can be assigned to specific domains. In order to efficiently insert modified Orb2 constructs into the endogenous locus we replaced most of the orb2 locus with the recognition site (attP) for the bacteriophage Φ C31 site-specific integrase (Fig.1A and Fig.S1). The Φ C31 integrase mediates unidirectional recombination between an attP and an attB site located in the targeting constructs. This system has previously been used to efficiently get targeted insertion of constructs in *Drosophila* (Bateman et al., 2006; Groth et al., 2004). While we were generating the orb2^{attP} transgenic flies, deep-sequencing methods suggested that the Orb2 locus contains three

extra isoforms (Orb2 E, F, and G) that would not be altered by our targeting strategy. Nonetheless, it is unlikely that these isoforms encode CPEB proteins in the classical sense, as they lack RBD domains.

The resulting transgenic flies, that replace the orb2 locus with an attP site, were homozygous lethal with only few escapees (TableS1). These escapees have strong locomotion defects and die soon after eclosion. Phenotypically, the observations match those seen for the previously published orb2^Δ null mutants (Keleman et al., 2007). The phenotype suggested that we had successfully modified the orb2 locus. The successful targeting was further confirmed by PCR and southern blot (Fig.1B-D).

Finally we tested the newly generated orb2^{attP} over a glutamine deficient orb2^{ΔQ} allele, which has previously been shown to lead to long-term memory defects in the courtship conditioning assay (Keleman et al., 2007). This allowed us to further test if orb2^{attP} was indeed a null mutant with respect to long-term memory. This heterozygous combination did not rescue long-term memory (LI=7.14, p=0.124, Fig.1F and TableS2) but did rescue the short-term memory (LI=27.16, p=0.0006, Fig.1G and TableS3). The learning index of orb2^{attP}/Orb2^{ΔQ} heterozygous flies matched the learning index of previously published orb2^Δ/orb2^{ΔQ} (Keleman et al., 2007) flies consistent with orb2^{attP} being a null mutant. Finally to validate our strategy, we used the ΦC31 integrase to reintroduce tagged wild type copies of orb2 back into the orb2^{attP} transgenic animals. The introduction of the tagged orb2^{GFP} was confirmed by RT PCR (Figure 1E). Orb2^{GFP} rescued flies do not have any obvious phenotypes. When tested for long-term memory, we found these alleles to fully restore the memory phenotype of orb2^{attP}/Orb2^{ΔQ} flies back to wild type level (LI=31.72, p<0.0001, Fig.1F and TableS2) confirming that Orb2 can tolerate a C-terminal GFP tag without functional impairment. In addition, we tested our novel GFP tagged glutamine deficient mutant (Orb2^{ΔQGFP}) allele that, like the previously published allele, showed normal short-term memory (LI=36.38, p<0.0001, Fig.1G and TableS3) but severe LTM defects when tested as homozygous (LI=1.35, p=0.388, Fig.1F and TableS2).

All these data suggest that replacement of the endogenous Orb2 coding sequence by an attP-site eliminates Orb2 function and can be used to insert any modification of interest into the endogenous orb2 locus.

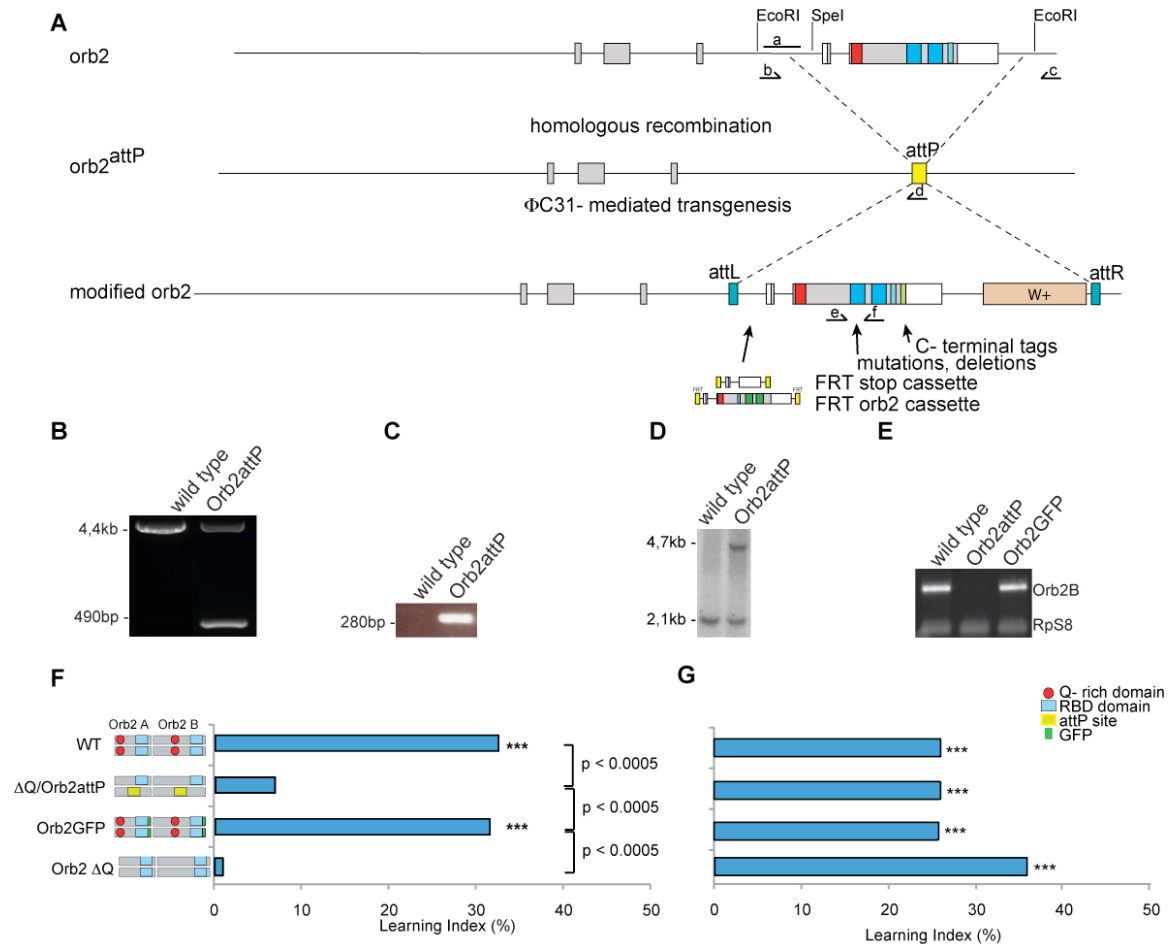


Figure 1 Generation and verification of Orb2attP and rescue constructs.

(A) strategy of *orb2^{attP}* generation and ϕ C31 mediated transgenesis to reinsert rescue constructs. (B) PCR verification using primers b and c (shown in A) results in a shift from 4.4kb in wt to 490bp in heterozygous mutants, confirming the desired deletion (C) PCR verification of the attP-site using primers b and d (shown in A) (D) Southern Blot verification using probe a (shown in A) after EcoRI/SpeI digest results in a shift from 2,1kb to 4,7kb for mutant bands in a heterozygous fly. (E) RT-PCR to confirm interruption of the transcript for *orb2^{attP}* homozygous mutant escapees and to confirm Orb2^{GFP} rescue flies using primers e and f (shown in A), RpS8: ribosomal protein used as internal reference control (F) Long term-memory assay of *orb2^{attP}* mutant when crossed to Orb2 ^{ΔQ} . The memory phenotype is fully rescued by Orb2^{GFP}, LI: Learning Index (G) Short-term memory assay of *orb2^{attP}* mutant when crossed to Orb2 ^{ΔQ} and for Orb2^{GFP} rescue. *P* values are

for $H_0: LI = LI_{Orb2GFP}$ (permutation test) and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for $H_0: LI = 0$ (permutation test)

3.2.2 Orb2 is enriched in the nervous system

In order to fully understand Orb2 function in long-term memory it is necessary to know when and where it is present to function. Of particular interest has been whether Orb2 is primarily pre- or post-synaptic. The mammalian counterpart of Orb2, mCPEB3, is specifically expressed post-synaptically (Huang et al., 2006), while AcCPEB1 is present in presynaptic compartments (Liu and Schwartz, 2003).

In situ hybridization experiments showed orb2 mRNA to be enriched in neurons and expressed throughout the entire adult brain, including expression in the mushroom bodies. In addition, tissue specific rescue showed that the primary function of Orb2 in long-term memory was within the mushroom bodies (Keleman et al., 2007). Despite the presence of α Orb2 antibodies in various labs (Si et al., 2010; Si et al., 2003a; Si et al., 2003b), only a single study to date addressed the expression of Orb2 protein. This study shows Orb2 to be present in cell bodies of embryonic neurons, but to be largely absent in axons. By contrast, in the adult brain Orb2 was shown to be present in axonal and dendritic terminals (Hafer et al., 2011). Rather than using α Orb2 antibodies we chose a different approach by tagging the endogenous protein. This allowed making use of commercially available α GFP antibodies. Importantly, as mentioned in the previous section, GFP tagged Orb2 is fully functional and thus likely to reflect the true expression pattern of the Orb2 protein.

In embryos Orb2^{GFP} was expressed in the embryonic nervous system (Fig.2A). In adult flies, Orb2 is abundantly expressed throughout the entire neuropil in brains and the VNC (Fig.2B). We did not observe Orb2 expression outside the nervous system at any of the observed stages (data not shown).

To determine whether Orb2 is expressed either in dendrites, axons or both we turned to mushroom body, a structure that is particularly suitable to differentiate between these compartments. They have well separated dendritic (calyx) and axonal (lobes) compartments. First, we saw that Orb2 is a purely cytoplasmic protein (Fig.2D and Fig.S2A+B), present in the soma and in axons. High-resolution images also showed Orb2 expression within the mushroom body calyx (Fig.2D and Fig.S2A+B). This means Orb2 is present in soma, axons and dendrites. Since dendritic and axonal areas reflect pre- and post-synaptic areas of a respective neuron, this suggested a presence of Orb2 in pre- and post-synaptic compartments.

In addition we observed a granular distribution of Orb2 throughout the entire neuropil, including soma, axons and dendrites (Fig.2D and Fig.S2A+B). Based on experiments from other groups it has been suggested that CPEB proteins of both classes (Bestman and Cline, 2009; Chae et al., 2010) and specifically Orb2 in cultured *Drosophila* neurons (Cziko et al., 2009) can localize to so called ribonuclear particles (RNPs) (Bestman and Cline, 2009; Chae et al., 2010; Cziko et al., 2009). We therefore, looked for co-localization of RNP markers with Orb2. When we tested the RNP markers FMRP, eIF4e, Me31B, Tral and DCP1 *in vivo* for co-localization at the MB calyx, we noticed that some of these RNPs indeed would co-localize with Orb2 (Fig.S2C-G). However, the high abundance of Orb2 made it impossible to statistically quantify the amount of overlap.

To establish the exact subcellular localization, we turned to immunoelectron microscopy, which clearly established a presence of Orb2 protein both in pre- and post-synaptic compartments (Fig.2E). In contrast to light microscopic analysis, we did not find Orb2 to be expressed in all neurons, but to be limited to some neurons of the MB calyx (Fig.2E)

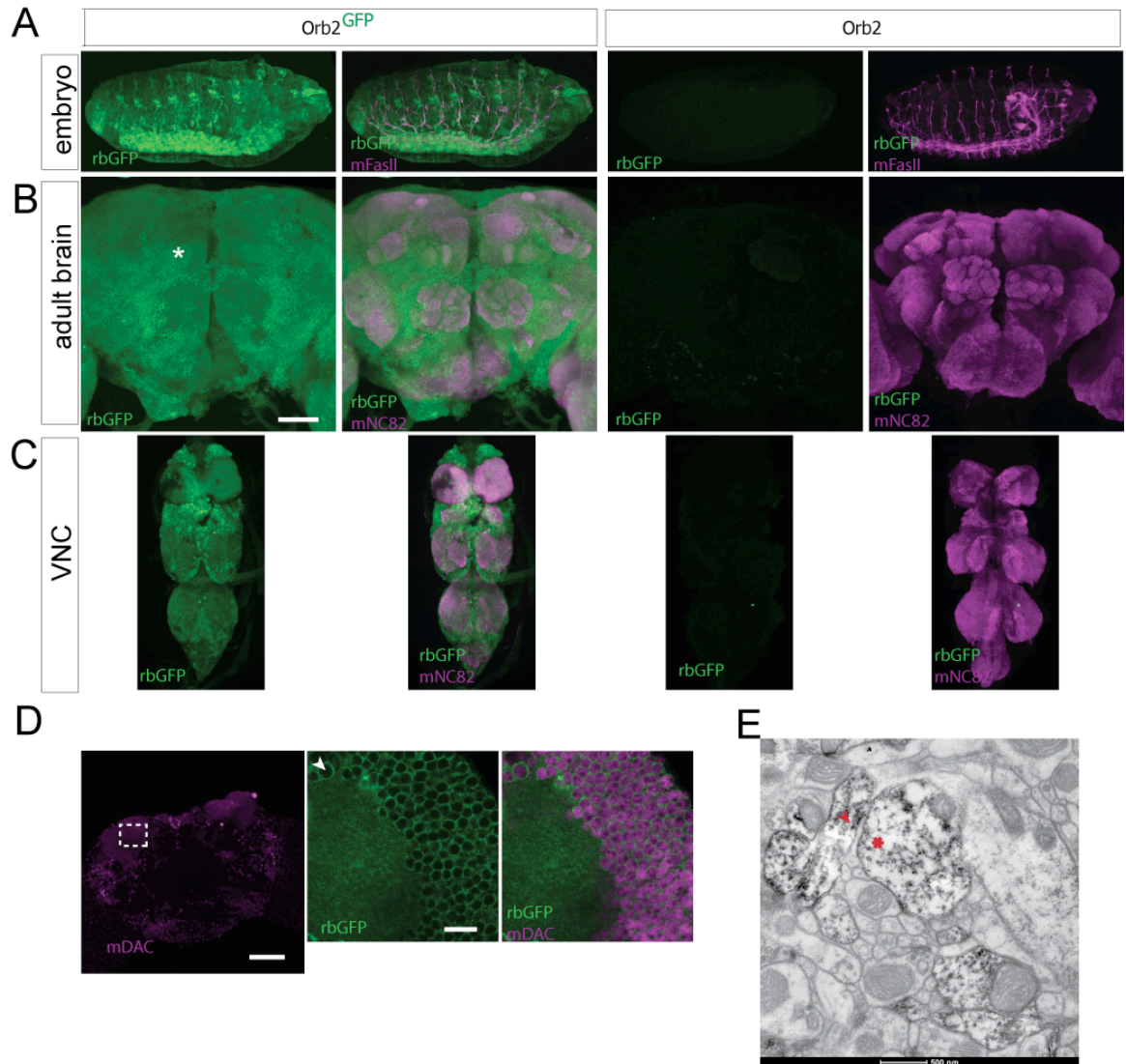


Figure 2 Expression pattern of Orb2.

(A) embryonic expression of Orb2^{GFP}. Orb2 is present in the nervous system only in Orb2^{GFP} animals but absent in Orb2 control animals. (B) Orb2^{GFP} is enriched throughout the entire brain only in Orb2^{GFP} animals but absent in Orb2 control animals. Asterix indicates the MBγ lobes (C) expression of Orb2^{GFP} in the adult VNC. Orb2 is present throughout the entire VNC in Orb2^{GFP} animals but absent in Orb2 control animals. VNC: ventral nerve cord (D) adult head stained with mDAC, a Kenyon cell marker. In the boxed area: close up of the MB Calyx, surrounded by its Kenyon cells. Arrowhead indicates the nucleus of a Kenyon cell, devoid of Orb2 staining (E), immuno electron microscopy shows Orb2^{GFP} to be a synaptic protein, present in pre- and postsynaptic compartment. Asterix indicate pre-, arrowhead indicates post-synaptic area. Scale bar: 50μm

3.2.3 Orb2 is required for neuronal development and function

Flies lacking the N-terminal glutamine stretch of Orb2 are viable but have a disrupted long-term memory (Keleman et al., 2007). By contrast, Orb2 deficient flies are lethal with few escapees that show a strong locomotion phenotype. Thus, Orb2 is likely to have pleiotropic functions. To test the affect on long-term memory of a complete Orb2 knock-out, we generated conditional knock-out alleles in which we flanked the Orb2^{GFP} rescue construct with FRT sites. Tissue specific FLPase expression can then selectively remove orb2^{GFP} in the context of a fly where all other tissues retain an unmodified orb2^{GFP} allele (Fig.1A). Orb2 has previously been demonstrated to be sufficient for long-term memory within the MB γ lobe (Keleman et al., 2007). The courtship promoting gene *fruitless* is highly enriched within this lobe (Keleman et al., 2007), therefore we used a previously published strain expressing the FLPase under the control of the fruitless promoter (Yu et al., 2010), to selectively remove Orb2^{GFP} from fru+ neurons. Selective removal of Orb2^{GFP} was confirmed by immuno-staining (Fig.3A). These flies had a dramatic decrease in general courtship (CI=20.94 $\pm$ 0.02, Fig.3B and TableS5). This suggested additional roles for Orb2 more generally in the fruitless positive cells outside the mushroom body, since removal of the MB neurons has previously been shown to block memory without a general impairment of courtship behavior (de Belle and Heisenberg, 1994).

To directly confirm Orb2 requirements in the MB for long-term memory, we selectively removed Orb2^{GFP} from the entire mushroom body, using the MB247Gal4 driver. Staining for GFP confirmed selective loss of Orb2^{GFP} from the mushroom body (Fig.3C). In contrast to Orb2^{GFP} removal throughout the fruitless positive cells, removal of Orb2^{GFP} from the MB resulted in a selective loss of long-term memory (LI=2.47, p=0.329, Fig.3D+TableS4) in comparison to control without a MB247Gal4 driver (LI=20.34, p=0.012, Fig.3D+TableS4). Finally, we attempted to confirm that MB γ lobe is the relevant MB lobe for courtship long-term memory. In order to accomplish this we employed a recently generated driver, VT29317 (unpublished Masser, Bidaye and Dickson) (Fig.S3) to express

FLPase specifically in the γ lobe. Although we observed only a very moderate decrease in Orb2^{GFP} expression within the MB γ lobe (data not shown), we found a significant decrease in long-term memory (LI=9.88, p=0.067 Fig.3D+TableS4) in comparison to control flies without FLP or heterozygous flies containing the non-FRT flanked orb2 allele, Orb2^{GFP} (LI=16.23, p=0.013 Fig.3D+TableS4). Interestingly, in addition to the long-term memory defect we also observed decreases in short-term memory (LI=6.45, p=0.117, Fig.3E+TableS5) in comparison to control without a Gal4 driver (LI=26.11, p<0.0001, Fig.3E and TableS5) or heterozygous cross to Orb2^{GFP} (LI=21.63, p=0.0004, Fig.3E and TableS5). Again, this result supports a model of more pleiotropic Orb2 function in neurons. Thus the role of Orb2 can be divided into two basic functions. First, it is required for development and/or the general function of neurons. This role does not require the N-terminal glutamine stretch since flies lacking this region do not have any obvious phenotype beside the long-term memory deficit. Second, it is acutely required during training for formation of a long-term memory. The latter function is critically dependent on the glutamine stretch.

In order to gain more insight into these two different functions of Orb2 and to further separate them we asked whether different Orb2 isoforms might explain the different function.

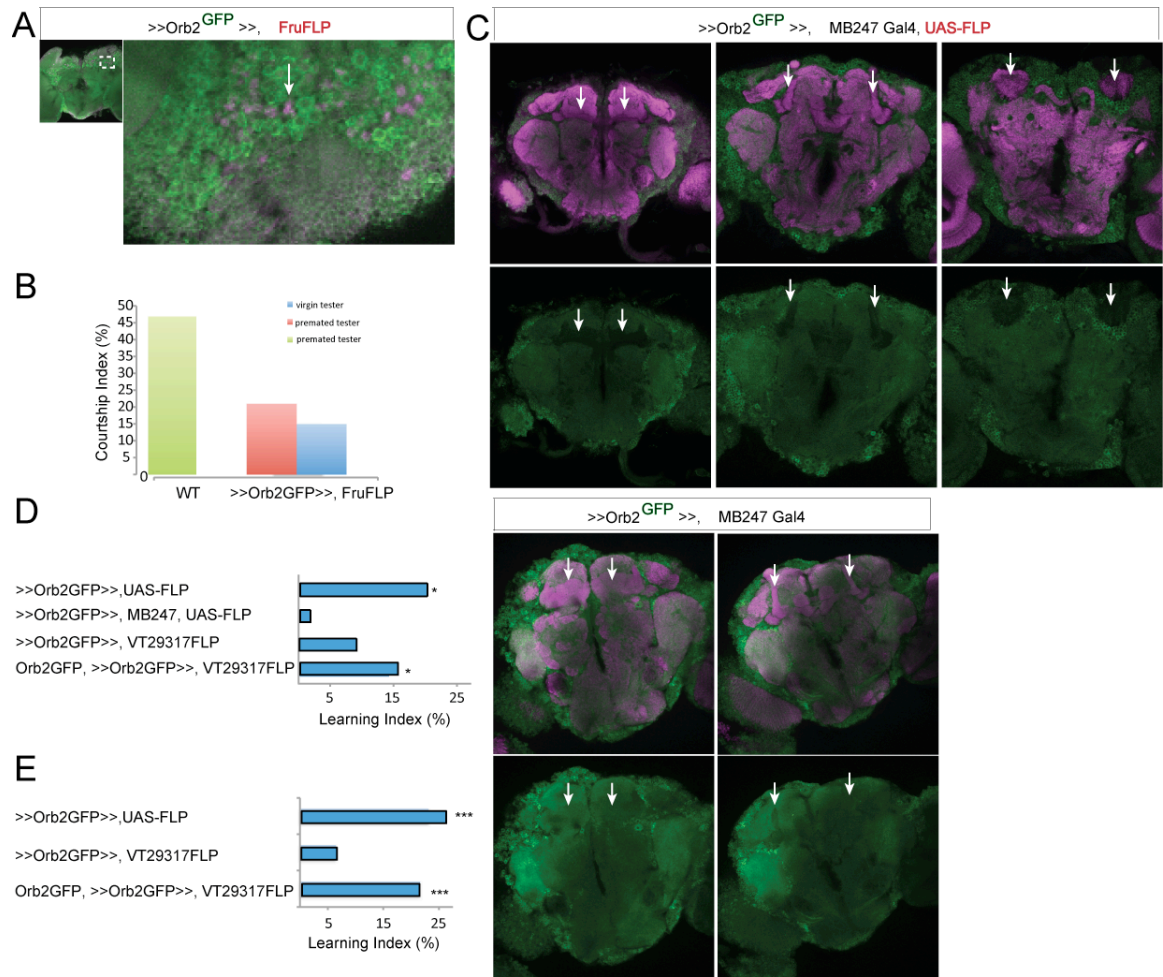


Figure 3 The FLP system

(A) FruFLP selectively removes Orb2^{GFP} from fru⁺ neurons. Arrows indicate fru⁺, orb2^{GFP} negative cells (B) Courtship Index of FRT flanked Orb2^{GFP} either co-expressing or without co-expression of FruFLP tested against virgins and predated females. (C) MB247 driven FLPase removes Orb2^{GFP} from the mushroom body. Arrows indicate absence of Orb2 in MB through optical slices (D) LTM assay after removal of Orb2 in mushroom body (E) STM assay after removal of Orb2 in mushroom body gamma lobe. * P<0.05, ** P<0.01, *** P<0.001 for H₀, LI = 0 (permutation test)

3.2.4 Orb2 isoforms have different functions during development and in long-term memory

The orb2 locus can potentially express five isoforms (Orb2 A, B, E, F, and G) (Fig.S4A). However only two of them, a shorter 60 kDa Orb2 A isoform and a longer 75 kDa Orb2 B isoform resemble the classical CPEB proteins with a glutamine-rich N-terminal region and a C-terminal RBD (Keleman et al., 2007; Si et al., 2003b). The Orb2 E, F, and G isoforms were identified by recent deep sequencing experiments. However, a recent report suggests that the isoforms E, F and G are rarely transcribed from the Orb2B promotor and vast majority of transcripts might be made from their own promotor (Hafer et al., 2011).

Transgenic expression of either Orb2A or Orb2B, using the UAS-Gal4 system (Brand and Perrimon, 1993), in the Orb2^{ΔQ} background can rescue the long-term memory phenotype (Keleman et al., 2007), suggesting these two isoforms are redundant. However, the GAL4-UAS expression system leads to overexpression, and thus subtle difference between Orb2A and Orb2B could possibly be overcome by virtue of the increased expression levels. Our orb2^{attP} allele allowed us to revisit this experiment at endogenous expression levels. Insertion of a single nucleotide in the isoform A specific exon leads to a frame shift, abolishing expression of isoform A but not isoform B (Fig.4A). Thus, Orb2^{ΔAGFP} should express only isoform B. These Orb2^{ΔAGFP} flies rescue lethality (TableS1) and locomotion phenotype of orb2^{attP} flies. Orb2^{ΔAGFP} expression pattern resembles that of Orb2^{GFP} (Fig.4B+D) and the protein can be readily detected by Western blot and Immunoprecipitation (Fig.4D and Fig.S7). Behaviorally, flies lacking the Orb2A isoform, orb2^{ΔAGFP}, have a severely disturbed long-term memory (LI=12.08, p=0.001, Fig.4F and TableS6). Nonetheless, a statistically significant memory remains in orb2^{ΔAGFP} flies. This result argues that the previous observation of redundancy was at least partially incorrect. The defect in memory is unique to long-term memory since short-term memory remains normal in orb2^{ΔAGFP} animals (LI=20.04, p<0.0001, TableS3). These results argue that the Orb2A isoform has a specific non-redundant role in

long-term memory formation. On the other hand, the rescue of lethality argues that the Orb2A isoform either does not function in development or alternatively is redundant with the Orb2B for developmental functions. These models can be distinguished by looking at the specific removal of OrbB. If flies expressing only Orb2A are viable, then Orb2A and Orb2B function redundantly in development.

Insertion of a single nucleotide in the isoform A specific exon and simultaneous removal of one nucleotide in the common exon leads to a frame shift of isoform B, abolishing expression of isoform B but not isoform A (Fig.4A). Thus, Orb2^{ΔBGFP} should express only isoform A. These Orb2^{ΔBGFP} flies are lethal (TableS1) and locomotion of escapees is severely impaired. Thus, Orb2B plays a specific non-redundant role in development. Due to the lethality, we could not directly test Orb2^{ΔBGFP} for a role in long-term memory. We therefore tested the Orb2^{ΔBGFP} allele over the Orb2^{ΔQGFP} allele, which by itself has no developmental defects but has severe long-term memory defects (Fig.4A). In this configuration flies are viable and the isoforms can be tested for long-term memory defects. Surprisingly, only Orb2^{ΔAGFP}/Orb2^{ΔQGFP} but not Orb2^{ΔBGFP}/Orb2^{ΔQGFP} flies have defects in long-term memory (LI=12.73, p=0.0003, and LI=12.08, p=0.001 respectively, Fig.4F and TableS6). However, as is the case for Orb2^{ΔAGFP} flies, Orb2^{ΔAGFP}/Orb2^{ΔQGFP} flies also still show some residual memory that is above that of the Orb2^{ΔQGFP} flies.

Because of the specific role for Orb2A in long-term memory we expected it to be expressed in adult brains. Unfortunately, we could not detect the Orb2A isoform by immuno staining of embryos or whole brains (Fig.4B) or VNC (Fig.4C). Also immunoprecipitation of Orb2^{ΔBGFP} or Orb2^{GFP} did not detect Orb2A protein (Fig.4D). Since we were puzzled by the apparent absence of the Orb2A protein despite its requirement for long-term memory formation we decided to perform isoform specific RT PCR. To confirm expression of both isoform-transcripts, wild type, orb2^{attP}, orb2^{GFP}, orb2^{ΔBGFP} and orb2^{ΔAGFP} flies were used in three independent RT-PCR experiments. As predicted, the isoform A specific primers amplified only transcripts from WT and orb2^{GFP} allele, whereas the mutant specific isoform A primers only amplified transcripts from the orb2^{ΔBGFP}

and orb2^{ΔAGFP} alleles. The isoform B specific primers amplified a product from all the constructs, except the null control. The null mutant served as a negative control and did not yield any products with any of the used primer combinations (Fig.4E). However, while these results suggest both transcripts to be present, recent deep sequencing data originating from the modENCODE project and our own unpublished results (Fig.S4B) indeed suggest the orb2A isoform transcript to be much less abundant compared to orb2B isoform. Reasons for this could either involve a more stringent transcriptional control of the Orb2A promoter or post transcriptional silencing, using the Orb2 3'UTR to inhibit translation, and/or force a restricted expression only in synapses. Other possible explanations for not detecting protein isoform A might be rapid degradation of the protein. But also a conformational inaccessibility for the antibody caused by a potential aggregation of this isoform, as suggested by (Si et al., 2010), might have prevented its detection.

An easy interpretation of the above results would be that during development only Orb2B, but not Orb2A, is required, while in long-term memory only Orb2A, but not Orb2B, is required. Thus, instead of being redundant, the isoforms would have unique roles in different processes and different developmental time intervals. However, such interpretation would predict that upon removal of Orb2A the entire memory would be lost, as is the case for Orb2^{ΔQGFP}. A simple way to reconcile these results would be to suggest that Orb2B might provide the residual long-term memory. However, the finding that Orb2^{ΔBGFP}/Orb2^{ΔQGFP} flies show completely normal long-term memory, argues against a simple model of non-redundant isoforms. Rather, it is likely that the function of Orb2B only becomes apparent when Orb2A is absent. Based on our findings of the orb2^{ΔQ} flies, this would be predicted to be dependent on the function of the Q-rich domain. By contrast, the finding that Orb2^{ΔBGFP}/Orb2^{ΔQGFP} flies show completely normal long-term memory, argues against a requirement of the Q-rich domain in Orb2B, and suggests the Orb2A Q-domain to be sufficient for long-term memory. Due to the developmental function of Orb2B, at this point we cannot address the requirements of Orb2B nor the sufficiency of Orb2A in

long-term memory. We can, however, address the discrepancy of these findings in a more precise way by generating isoforms specific deletions of the Q-rich domain.

3.2.5 The Q-rich domain is necessary and sufficient in Orb2A and dispensable in Orb2B for long-term memory

For analysis of Orb2 isoforms lacking the Q-rich domain, we followed a similar strategy as outlined in 3.2.4 and additionally deleted the Q-rich domain.

Similar to what we had seen previously with Orb2^{ΔAGFP}, Orb2^{ΔAΔQGFP} flies were viable (TableS1), displayed no locomotion defects and the protein seemed to be normally expressed (Fig.S5A+B). Also consistent with what we had seen previously for Orb2^{ΔBGFP}, Orb2^{ΔBΔQGFP} flies were lethal (TableS1) and the few escapees had no detectable level of the Orb2A protein (Fig.S5A+B). Thus, as we had previously seen, the Orb2B isoform is expressed at higher levels than the Orb2A isoform and in general this expression does not depend on the Q-domain.

To confirm the behavioral requirement of the glutamine stretch in long-term memory for both isoforms we made use of a similar strategy as above. We tested the isoform specific glutamine deficient alleles Orb2^{ΔBΔQGFP} and Orb2^{ΔAΔQGFP} over Orb2^{ΔQGFP}. No rescue of long-term memory was found in both cases (LI=7.25, p=0.053 and LI=1.35, p=0.418 FigS5C and TableS7). This was expected since no Q-rich domain was present in this combination. The same was true for the homozygous viable Orb2B glutamine deficient allele Orb2^{ΔAΔQGFP} (LI=2.17, p=0.367 Fig.4F and TableS6). Thus the residual Orb2B dependent memory in the absence of Orb2A is also dependent on the Q-rich domain, arguing for a function of Orb2B in long-term memory in addition to its developmental role. However, this might still be explained by a partial redundancy in isoform function upon removal of Orb2A. To assign the function of the glutamine domain specifically to each isoform we therefore had to test one isoform with a deletion of the Q-rich domain complemented with the other wild type isoform.

First, as a control we tested whether the two isoform specific wild type alleles are at all able to complement each other's function. Indeed we observed a rescue of long-term memory (LI=18.39, $p<0.0001$ Fig.4F and TableS6), albeit not to wild type levels. Since this combination expresses only one half of the wild type levels for each isoform we suggest that both isoforms function in a dosage dependent manner, as published in our previous work (Keleman et al., 2007).

Next, we tested isoform specific deletion of the Q-rich domain complemented by the other isoform without a deletion of the Q-domain. Interestingly, only Orb2^{ΔAΔQGFP} (LI=16.97, $p=0.001$ Fig.4F and TableS6) but not Orb2^{ΔBΔQGFP} (LI=2.86, $p=0.275$ Fig.4F and TableS6) could rescue the long-term memory defect when crossed to the other isoform. This strongly suggests that the Q-rich domain is dispensable in Orb2B and required and sufficient for Orb2A function in long-term memory, in cases where both isoforms are present.

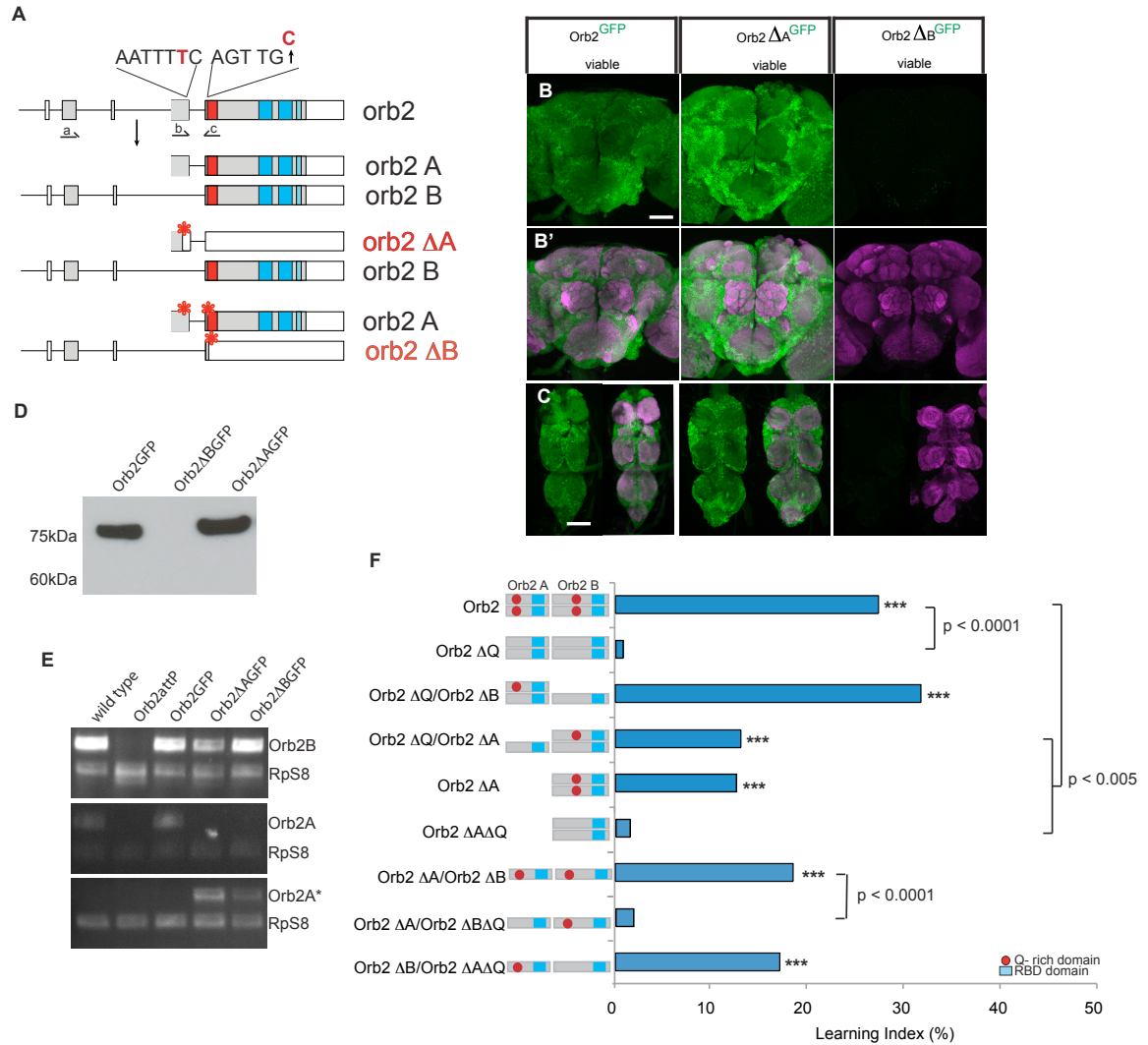


Figure 4 Isoform specific rescues.

(A) Scheme for generation of isoform specific rescues. Insertion of one nucleotide in the A specific exon generates Orb2^{ΔAGFP}. Insertion of one nucleotide in the A specific exon with subsequent removal in the common exon generates Orb2^{ΔBGFP}. (B) Adult brain immuno staining of GFP tagged isoforms and (B') GFP and NC82 overlap. (C) VNC immuno staining of GFP tagged isoforms and NC82 overlap. Scale bar: 50 μm (D) IP of Orb2^{GFP}, Orb2^{ΔAGFP}, Orb2^{ΔBGFP} from adult heads. No Orb2A can be detected in any of these constructs. IP: Immuno precipitation, WB: western blot (E) RT-PCR confirms the presence of orb2A transcript (primer b and c as shown in A), modified Orb2A and Orb2B transcript (primer b* and c as shown in A) and Orb2B transcript (primer a and c as shown in A) (F) LTM assay of isoform specific alleles suggests the Q-rich domain to be specifically required for Orb2A function and to be dispensable for Orb2B function. *P* values are for H_0 : $LI = LI_{Orb2GFP}$ (permutation test) and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for H_0 , $LI = 0$ (permutation test)

3.2.6 Homologous Q-domains can functionally substitute the Orb2 Q-domain in LTM

Our previous work (Keleman et al., 2007) had suggested that the N-terminal of Orb2 is dispensable for development but required for long-term memory. However, the features of the N-terminal glutamine-rich domain were not analyzed. Does the Orb2 N-terminal, for instance, have a unique non-conserved function, as might be suggested from the lack of sequence conservation, or is it rather just the presence of multiple glutamines within a non-conserved domain that provide the functions for long-term memory? To address this we generated chimeras between the CPEB family members and Orb2.

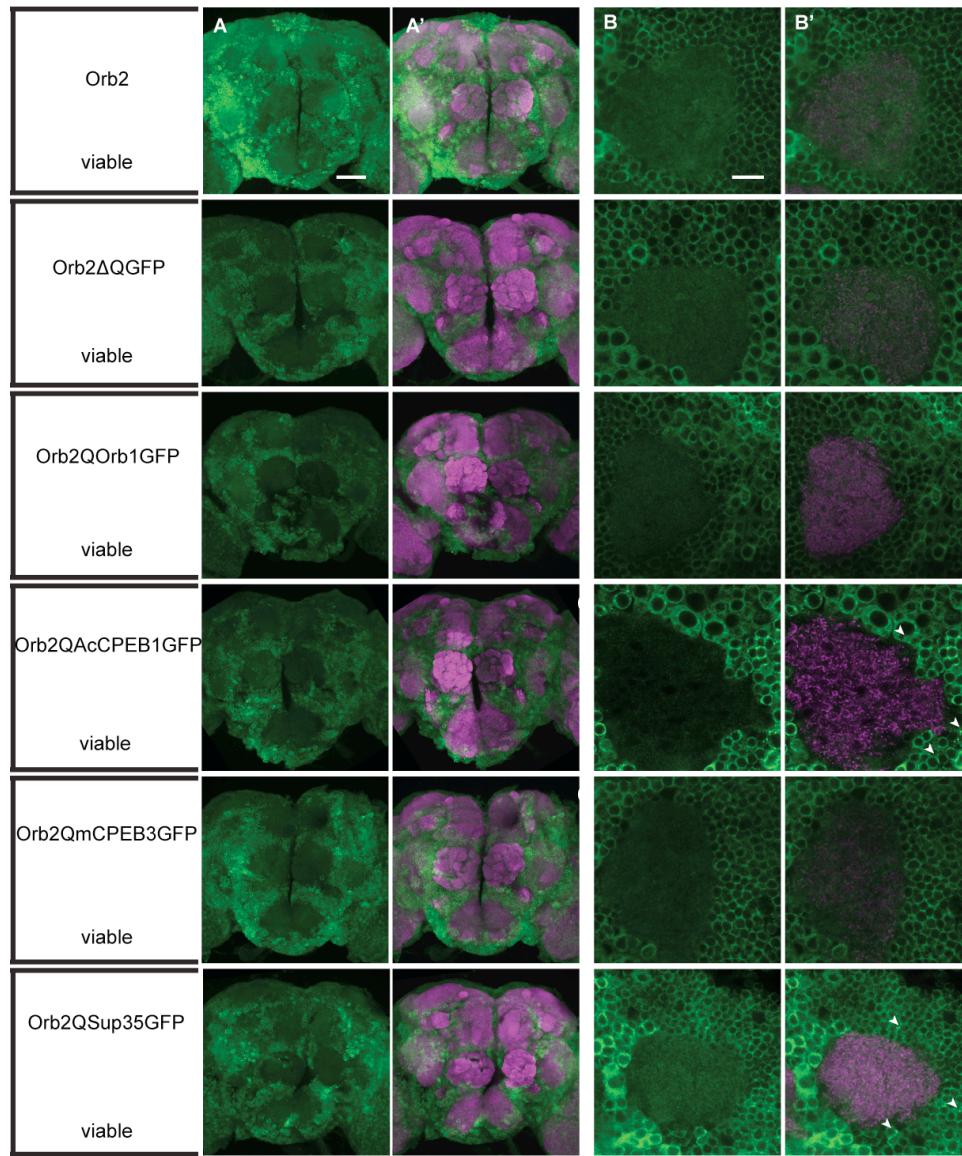
Many CPEB proteins have a low complexity region, enriched in glutamine residues, in their less conserved N-terminus. However, both length and content of these Q-stretches vary considerably among different CPEB members. The AcCPEB1, Q-stretch has been suggested to have prion-like properties (Si et al., 2010; Si et al., 2003a; Si et al., 2003b). Surprisingly, it is in the aggregated prionic state that AcCPEB1 is active in mRNA translation (Si et al., 2003b).

By using our newly generated orb2^{attP} allele we reproduced the previous observation that the Q-rich domain has a specific role in long-term memory. Insertion of an Orb2 lacking the Q-domain, Orb2^{ΔQGFP}, rescues lethality and locomotion but has a specific deficit in long-term memory (Fig.1G and TableS2). In addition, Orb2^{ΔQGFP} brains did not show any gross morphological nor obvious mislocalization of Orb2 protein (Fig.5A +Fig.S7). This indicates that the Q-domain is neither necessary for development nor for the localization or stability of the protein.

The Q-domains of the CPEB proteins Orb1, mCPEB3 and AcCPEB1 as well as the yeast prion SUP35 were cloned to replace the endogenous Orb2 glutamine stretch (Fig.5A). We did not observe any obvious alteration in protein localization (Fig.5A+B) or levels of the chimeric proteins (Fig.S7) and also the general development seemed to be undisturbed. However, for Orb2^{Qsup35GFP} and Orb2^{QAcCPEB1GFP} we did notice a more pronounced punctate expression pattern

(Fig.5B). Surprisingly Orb2^{QAcCPEB1GFP} (LI=19.89, p<0.0001, Fig.5C and TableS8) and Orb2^{QmCPEB3GFP} (LI=19.86, p=0.04 Fig.5C and TableS8) flies had full rescue of the long-term memory defect of Orb2^{ΔQGFP} flies, while Orb2^{Qorb1GFP} (LI=9.16, p=0.028 Fig.5C and TableS8) and Orb2^{Qsup35GFP} (LI=10.23, p=0.023 Fig.5C and TableS8) had only a partial rescue. Interestingly, AcCPEB1 (Heinrich and Lindquist, 2011; Si et al., 2010; Si et al., 2003b) and mCPEB3 (personal communication E.Kandel) have been implicated to act at least in parts in a prion-like manner.

Together these results imply that the exact sequence of the N-terminal domain is important. These domains have been suggested to fold into β -sheet rich aggregating structures (Alberti et al., 2009; Wickner et al., 2007), thereby conferring prionic properties to the protein. However, a more recent report suggests that in addition to β -sheets, these domains might form α -helical coiled-coil structures (Fiumara et al., 2011), which allows interaction with proteins carrying similar structures to facilitate aggregation. To test such a possibility for Orb2 we tested coiled-coil predictions. Indeed the Orb2 Q-rich domain is also predicted to form coiled-structures (Fig.S6). However, such a prediction is also true for the Q-domain of Orb1 and Sup35, which failed to rescue the long-term memory phenotype. By contrast the Q-domain of mCPEB3, which could rescue the long-term memory phenotype is not predicted to form coiled-coils (Fig.S6). Therefore it seems unlikely that the Q-domain of Orb2 functions simply by formation of coiled-coils. However, further mutations in the heptad repeats, required for coiled-coil formation should answer this in more detail.



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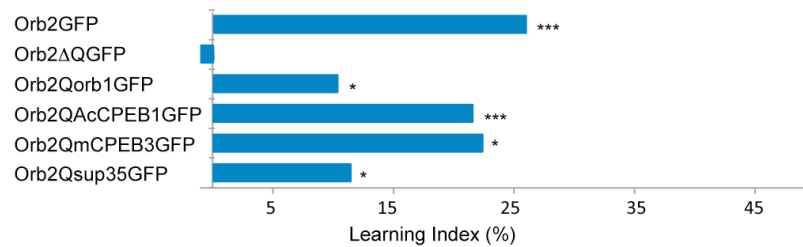


Figure 5 Replacement of the Orb2 Q-rich domain.

(A) immuno staining of GFP tagged adult heads, GFP channel (A') GFP and NC82 overlap (B) MB calyx close up of GFP tagged adult heads, GFP channel (B') GFP and NC82 overlap. Arrowhead indicates enlarged spots of Orb2. Scale bar: 50 μ m (C) LTM assay of Orb2 chimeras with replaced Q-domains. *P* values are for H_0 : $LI = LI_{Orb2GFP}$ (permutation test) and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for H_0 , $LI = 0$ (permutation test)

3.2.7 Homologous RBDs of the mCPEB2-4 subfamily can substitute the Orb2 RBD in development

Our previous results suggest the Q-rich domain is sufficient in Orb2A and seems to be dispensable in Orb2B for long-term memory, at least in cases when both isoforms are present. To address whether isoform specific requirement also applies to the RBD of Orb2 we analyzed this domain in more detail. The best understood difference within the two different subfamilies of CPEB proteins lies within their RBDs. Members of both subfamilies show a remarkable sequence conservation of the RBD within their subfamily (>87%) while there is not so much similarity between the families (40-45%) (Mendez and Richter, 2001), suggesting different target specificity. Much is known about the mechanisms and targets of the RBD domain in CPEB1 subfamily, where it binds the 3' UTR cytoplasmic polyadenylation element (CPE; consensus UUUUUAU) of target mRNAs (Hake and Richter, 1994) to control their polyadenylation (Mendez and Richter, 2001). Both RRM and the Zinc finger are required for binding to CPEs with high affinity (Hake et al., 1998). In contrast almost nothing is known about the mechanism and targets of the RBD of the second subfamily. It has been suggested that mCPEB2-4, unlike CPEB1, do not bind CPE elements but might recognize tertiary RNA structures of their target mRNAs (Huang et al., 2006). However, it remains unclear whether the various members of this family recognize similar or distinct features in their target mRNAs.

First, to define the role of the RBD we generated chimeric Orb2 constructs replacing its native RBD by RBDs of Orb1, mCPEB1, AcCPEB1, mCPEB2, 3 and 4 (Fig.S8C). All Chimeras with RBDs of the CPEB1 subfamily resulted in homozygotic lethal flies (TableS1) with only few escapees. Immuno staining of the few escapees showed that the lethality was not a result of lack of protein expression (Fig.S8D and Fig.S7). High resolution imaging of the MB calyx confirmed these results (Fig.S8E). Lethality suggested that these flies are not able to bind and regulate Orb2 targets anymore. This confirmed the expected differential target specificity of the two CPEB subfamilies.

By contrast, chimeric proteins with RBDs of the CPEB2 subfamily were viable (TableS1) and showed normal locomotion behavior as well as normal courtship behavior (TableS9). Expression of the chimeras appeared largely normal in these animals (Fig.S8D and Fig.S7). Nonetheless, *orb2*^{mCPEB2RBDGFP} (LI=9.25, p=0.012 Fig.S8B and TableS9), *orb2*^{mCPEB3RBDGFP} (LI=5.69, p=0.181 Fig.S8B and TableS9), and *orb2*^{mCPEB4RBDGFP} (LI=9.87, p=0.088 Fig.S8B and TableS9) still all have long-term memory defects. By contrast the short-term memory of *orb2*^{mCPEB2RBDGFP} appeared to be normal (LI=42.34, p<0.0001 Fig.S8C and TableS11), arguing that the long-term memory phenotype is not just due to a hypomorphic nature of the *orb2*^{mCPEB2RBDGFP} allele. Thus this result suggests that indeed the RBD domain is critically required for Orb2 function in long-term memory, most likely for local translation of mRNA specifically at neurons. This function seems to be different from the developmental function. Because the RNA binding domain largely specifies the mRNA targets of CPEB proteins, this argues that the mRNA required for long-term memory might not be very well conserved between *Drosophila* and mouse.

At this point we do not know which isoform would require its RBD for long-term memory. However, the above finding for the first time allowed us to directly dissociate the developmental function of Orb2B from its function in long-term memory.

3.2.8 The RBD of Orb2B is required for long-term memory

To test whether both isoforms would equally require its RBD we made use of above finding that replacement of the endogenous Orb2 RBD by mCPEB class II RBD does rescue the development but not the long-term memory. We used these flies and tested them over either the Orb2A or Orb2B isoform.

The long-term memory defect of *Orb2*^{mCPEB2RBDGFP} could be fully rescued when crossed to the Orb2B expressing allele *orb2*^{ΔAGFP} (LI=27.33, p<0.0001 Fig.6A and TableS10) but not when crossed to the Orb2A expressing allele *orb2*^{ΔBGFP} (LI=-9.69, p=0.699 Fig.6A and TableS10), suggesting that the RBD in

Orb2B is required, while the RBD of Orb2A might be dispensable for long-term memory. However, while Orb2^{mCPEB2RBDGFP} crossed to Orb2B has normal courtship behavior, the cross to Orb2A has severely reduced courtship levels (TableS10). This might be due to the fact that the latter cross expresses only half the dosage of RBD that can function during development in comparison to Orb2^{mCPEB2RBDGFP} alone or when crossed to Orb2B. However, since a single copy of Orb2B is sufficient for development, this suggests that mCPEB2 RBD functions differently to Orb2 RBD both in development and long-term memory.

In summary these results suggest that the Orb2B RBD is required to regulate target mRNAs during both, the development and the memory formation, while the Orb2A RBD seems not to be required for both processes. However, the developmental limitations of this experiment require further confirmation that the Orb2A RBD is indeed dispensable for long-term memory.

3.2.9 The RBD of Orb2A is dispensable for long-term memory

The lack of long-term memory rescue with RBD chimeras precluded identification of the relevant residues in the RBD. To identify the relevant residues we generated point mutations within the Orb2 protein.

The RBD of Orb2 contains two RNA recognition motifs and a Zinc finger. We designed mutants with point mutations in either RRM1 or the Zinc finger, which was predicted to prevent RNA binding, based on homologues alignments with other RNA binding proteins (Fig.S8A). Point mutations in either RRM1, RRM2 or the zinc finger cannot rescue lethality (TableS1), and few homozygous escapees show a strong locomotion defect, although these mutations do not modify the overall protein localization or expression levels (Fig.S8D+D and Fig.S7). CLIP (UV- crosslinking and Immunoprecipitation) experiments performed in our lab with these mutations show no binding to RNA in case of point mutations in RRM motifs, whereas mutations in the Znf do not prevent RNA binding (Stepien and Keleman unpublished). This argues for a role of the RRM in

general RNA binding where the Znf might contribute to the specificity of this binding, similar to observations in mouse (Huang et al., 2006).

Due to the lethality of the RBD mutation we tested these alleles for a role in long-term memory by crossing them to Orb2^{ΔQGFP}. We reasoned that in such a cross with both alleles encoding unfunctional proteins, we would not be able to rescue the long-term memory defect. Strikingly, we found these flies to rescue the LTM defect (LI=25.48, $p < 0.0001$ Fig.6B and TableS12).

On the first glance this might argue against a function of the RBD in long-term memory formation and thus would not fit the hypothesis of Orb2 as a regulator of RNA translation in long-term memory. Because the previous experiment suggested Orb2B RBD to be critically required for long-term memory it is possible that the two isoforms are able to complement each other. To test this hypothesis we looked at the memory of RBD mutated alleles crossed to either isoform. Orb2A crossed to the RBD mutant could not rescue the lethality, arguing again for a strong requirement of the RBD domain in Orb2B's developmental function. However, Orb2B crossed to RBD mutations was able to rescue the long-term memory phenotype (LI=17.31, $p < 0.0001$ Fig.6B and TableS12). This would suggest the RNA binding domain to be dispensable for Orb2A function. To further address this possibility we again generated isoform specific mutations of the RNA binding domain, following the same strategy as outlined before in 3.2.4.

Flies expressing only Orb2B with a mutated RBD (referred to here as orb2^{ΔARBD*GFP} allele) are lethal and when crossed to Orb2A with a functional RBD the lethality is not rescued. This suggests that the Orb2A RBD is not sufficient in development and that it is the Orb2B RBD, which is required for the developmental function of Orb2B.

Flies expressing only Orb2A with a mutated RBD (referred to here as orb2^{ΔBRBD*GFP} allele) are lethal as well, due to removal of Orb2B. However, when crossed to Orb2B with a functional RBD both the development and the long-term memory (LI=17.96, $p = 0.0001$ Fig.6B and TableS12) are rescued. This suggests that the RBD is required and sufficient in Orb2B, but dispensable in Orb2A both

during development and in long-term memory. In addition when crossed to Orb2B lacking the Q-rich domain these flies fully rescue the long-term memory defect (LI=15, $p=0.005$ Fig.6B and TableS12), supporting the earlier finding that the Q-rich domain is dispensable in Orb2B for long-term memory. Thus we believe that Orb2A and Orb2B complement each other in long-term memory formation.

Such allelic complementation has previously been used to suggest that a protein might function as a complex (Carlson et al., 1981; McMurry and Levy, 1995; Zhang et al., 2006). This would fit nicely with the proposed Q-domain dependent aggregation of Orb2. However, two different models could achieve such a complementation: in model 1) Orb2A and Orb2B would directly interact with each other, whereas in model 2) Orb2A and Orb2B might function either independently from each other or mediate each other's function with the help of other proteins. Recent findings in *Aplysia* suggest that indeed CPEB proteins are able to form prion-like self-aggregates, which would clearly favor model one (Heinrich and Lindquist, 2011; Si et al., 2010).

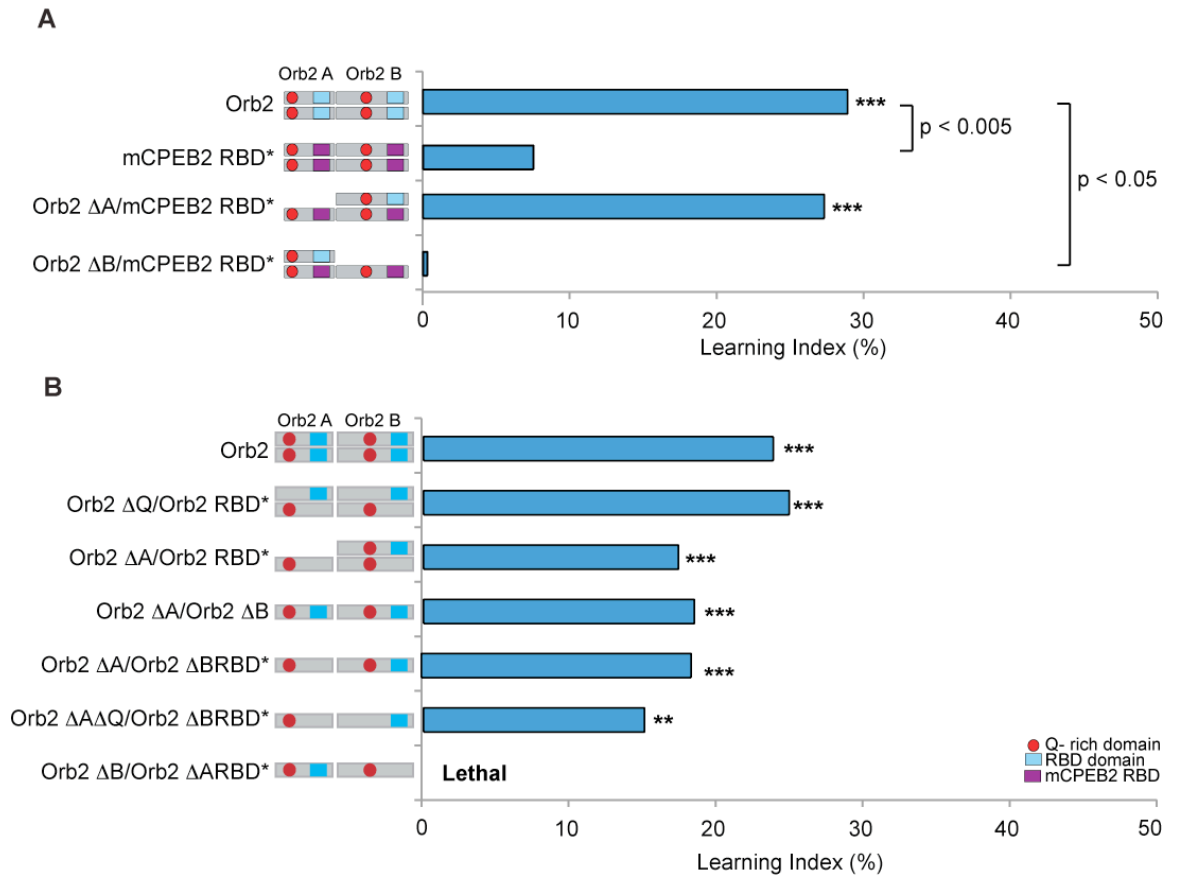


Figure 6 The RBD is required in Orb2B but dispensable in Orb2A for long-term memory.

(A) LTM assays of flies in which the RBD has been replaced by homologous mCPEB2 RBD. Replacement of the endogenous Orb2 RBD does rescue lethality but not LTM of Orb2^{mCPEB2RBD} flies (B) LTM assays of flies with point mutations in Orb2A and Orb2B RBD suggest the Orb2A RBD to be dispensable for LTM function. *P* values are for H_0 : LI = LI_{Orb2GFP} (permutation test) and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for H_0 , LI = 0 (permutation test)

3.2.10 Orb2 forms high molecular weight complexes

Orb2A and Orb2B might have independent non-redundant roles in long-term memory. Substitutions with Q-domains from various CPEB proteins suggested that it probably is not a unique sequence within the Q-domain but rather the enrichment in the negatively charged glutamine residues that is the key factor of the Q-domain. In other proteins like mCPEB3 and AcCPEB1 this domain has been proposed to confer prionic properties to the protein (Heinrich and Lindquist, 2011; Si et al., 2010). Because prions have the property of forming

aggregates when switched into a prionic state, we tested the aggregation properties of Orb2 constructs expressed in *Drosophila* S2 cells. Expression in S2 cells provides a level of resolution that could not be achieved in whole brains due to the small size of *Drosophila* neurons and the large number of densely packed cells.

We found that only Orb2A forms visible punctuates in S2 cells. Punctuate formation is greatly reduced when the Q-domain is deleted from Orb2A (Fig.7A). Orb2B did not show any punctuate pattern (Fig.7A). However, Orb2A is expressed at very limited amount in *Drosophila* nervous system and thus punctuate formation of Orb2A could very well be artefactual due to protein over expression in S2 cells. Interestingly, when we analyzed S2 cell lysates by SDD-AGE analysis, both, Orb2A and Orb2B, formed large complexes, which were resistant to SDS treatment. These complexes were again dependent on the Q-stretch, since its deletion markedly reduced complex formation (Fig.7A).

To exclude artefactual aggregation due to over expression and to test aggregation in the *in vivo* system we performed immunoprecipitation experiments of Orb2^{GFP} from adult heads, using large amounts of fly heads to overcome possible limitations in detection by low level expression and/or aggregation of Orb2A. Indeed, when immunoprecipitated with GFPtrap and probed in Western Blot using either α GFP or an antibody generated against Orb2A, α Orb2 273 (unpublished Si K), we detected bands migrating much higher than the previously described monomeric Orb2B. These bands migrate on SDS gels at reducing conditions between 214kDA and 240kDA (Fig.7C+D). Since we were able to probe them with two different antibodies we are sure that these bands do reflect oligomeric Orb2 proteins and thus might be the aggregates observed in S2 cells.

Next we wanted to know whether *in vivo* there would be any difference in the Orb2 isoforms ability to form these complexes. To do so we performed immunoprecipitation experiments of Orb2^{GFP} and compared them to isoform specific pull downs. Interestingly, removal of Orb2A leads to a significant decrease in Orb2B aggregation, when compared to wild type flies. By contrast, most of the Orb2A protein seems to exist in an oligomeric state (Fig.7E). This

suggests that possibly very little of the Orb2A present in neurons is sufficient to bring Orb2B into complex. At this point, however, we failed to prove any direct interaction between the two isoforms. This might be due to the very low expression level of Orb2A. Alternatively additional proteins might be involved in this process and present in this complex.

In contrast to findings in S2 cells we could not observe a decrease in oligomerization upon removal of the Q-domain in the brain (Fig.7F), despite the fact that such a mutation has severe long-term memory defects. However, isoform specific biochemical analyses regarding the Q-domain still need to be done.

In summary we have shown that Orb2 is able to form oligomers *in vivo* and that it is likely to be the Orb2A isoform, which would initiate this process.

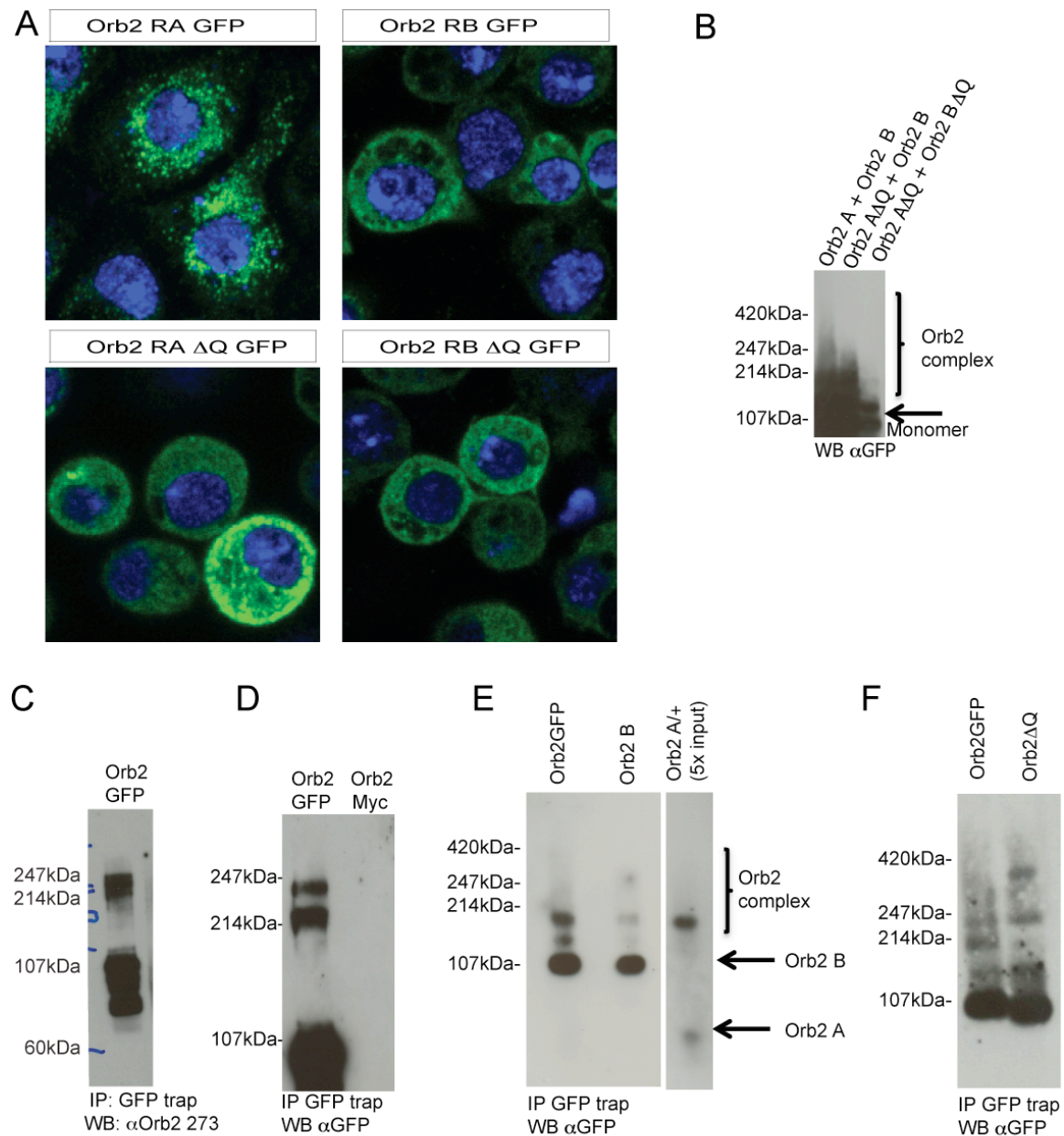


Figure 7 Orb2 forms high molecular weight complexes.

(A) immuno staining of S2 cells overexpressing Orb2 RA , RB, ΔQRA, ΔQRB tagged with GFP, (B) Lysates of double transfected cells probed for Orb2BGFP (C) IP of Orb2^{GFP} from adult heads probed against αOrb2 273 on SDS-gel (D) IP of Orb2^{GFP} and Orb2^{Myc} from adult heads probed against αGFP on SDS-gel (E) SDD-AGE analysis of Orb2 isoforms, IP of Orb2^{GFP} from adult heads probed against αGFP (F) SDD-AGE analysis of Orb2^{ΔQGFP} from adult heads probed against αGFP.

3.2.11 The Orb2 3'UTR is required for Orb2 function

Aside from the RBD and the Q-domain, Orb2 does not contain any defined regions. However, the orb2 3' UTR has been suggested to contain binding sites for numerous miRNAs as well as for numerous other regulatory proteins (Morgan et al., 2011). To test a possible function of the orb2 3'UTR in long-term memory we replaced the endogenous 3' UTR by SV40 3'UTR.

Flies carrying the modified Orb2^{SV40 3'UTRGFP} allele were viable. However, these flies did have very low courtship levels (TableS13). This was not due to decreases in expression or stability of Orb2 as the staining showed rather up-regulation of Orb2 by immuno staining of adult brains (Fig.8A) and immunoprecipitation experiments (Fig.8B). When tested for long-term memory these flies showed a severe long-term memory defect (LI=0, p=0.501 Fig.8C and TableS13). This might however be due to the observed reduced courtship level of the respective males rather than a specific long-term memory defect.

To test whether both isoforms equally depend on the function of their 3'UTR, we again generated isoform specific replacement of the endogenous 3'UTR by SV40 3'UTR following the similar strategy outlined before in 3.2.4.

Similar to what we observed for Orb2^{SV40 3'UTRGFP}, Orb2B with SV40 3'UTR (Orb2^{ΔA SV40 3'UTRGFP}) resulted in reduced courtship levels and a long-term memory phenotype when crossed to Orb2A (LI=-1.28, p=0.589 Fig.8C and TableS13). Also similar to what we observed for Orb2^{SV40 3'UTRGFP}, Orb2B with SV40 3'UTR flies showed an increase in protein in immuno stainings (Fig.8A) and in immunoprecipitation experiments (Fig.8B)

Like in previous experiments, flies expressing only Orb2A with SV40 3'UTR (Orb2^{ΔB SV40 3'UTRGFP}) were lethal (TableS1). When crossed to Orb2B, to overcome the lethality, these flies showed a severe long-term memory phenotype (LI=-3.09, p=0.693 Fig.8C and TableS13), but normal courtship levels (TableS13). Although we were able to observe an increase in protein level of

Orb2B protein for both Orb2^{SV40 3'UTRGFP} and Orb2^{ΔA SV40 3'UTRGFP}, a potential increase of Orb2A protein level was not sufficient to detect the protein in immuno staining (Fig.8A) or immunoprecipitation experiments of adult heads (Fig.8B).

In summary the Orb2 3'UTR is required for the function of both isoforms. In Orb2B it seems to down regulate the amount of protein and when interfered with this function the development seems to be disturbed, resulting in reduced courtship levels. In general the 3'UTR is required for the developmental function of Orb2B and the long-term memory function of Orb2A.

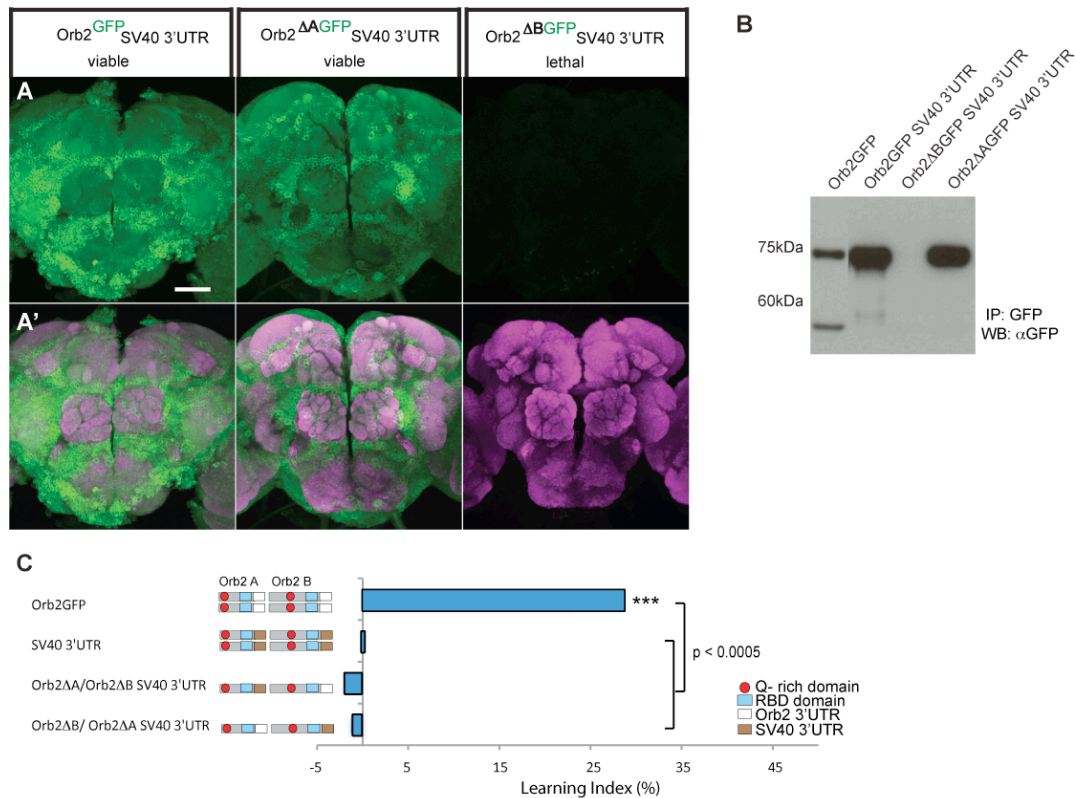


Figure 8 The Orb2 3'UTR is required for Orb2 function.

(A) immuno staining of adult brains against GFP and (A') GFP and NC82 overlap in which the endogenous 3'UTR was replaced by SV40 3'UTR. (B) IP experiments show upregulation of Orb2B expression upon 3'UTR exchange (C) LTM assay of flies in which the endogenous 3'UTR was replaced by SV40 3'UTR. *P* values are for $H_0: LI = LI_{Orb2GFP}$ (permutation test) and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for $H_0, LI = 0$ (permutation test)

3.3 Summary and conclusions

3.3.1 Successful insertion of attP into the Orb2 locus allows endogenous modification of Orb2

Orb2 was recently shown to be specifically required for long-term memory during the time of training but to be dispensable for short-term memory and recall (Keleman et al., 2007). To elucidate the molecular mechanism of Orb2 function in long-term memory we generated a novel allele in which we replaced most of the orb2 ORF with an attP acceptor site (Fig.1A). This tool allowed us to rapidly modify the endogenous Orb2 protein and perform a structure function analysis. We then used Φ C31-mediated site directed mutagenesis to reinsert a GFP-tagged rescue construct (Fig.1A and Fig.S1) to verify our strategy. Behavioral tests confirmed this construct to fully rescue the observed orb2 knock-out phenotypes (viability, locomotion, LTM) (Fig1), excluding any influence of the tag on Orb2 function. Thus this new allele, orb2^{attP2} allowed us to conduct a large-scale structure/function study, where functional differences could be unambiguously ascribed to specific regions of the protein.

3.3.2 Orb2 is a neuronal gene expressed in pre- and post synaptic compartments

Using the new Orb2^{GFP} we have analyzed the expression pattern of the Orb2 protein throughout various developmental stages. Orb2 is expressed from onset of the embryonic nervous system development to adult stage (Fig.2). Orb2 is a purely cytoplasmic protein, consistent with its predicted role for cytoplasmic translational control (Huang et al., 2006).

In adult brains Orb2 seems to be expressed in the cell body, dendrites and axons. To elucidate the sub-cellular localization we used immuno electron

microscopy and found Orb2 to be present both in pre- and post- synaptic compartments. This is in contrast to findings for the mammalian homologue CPEB3 that was shown to only act post-synaptically and to AcCPEB1 that was shown to be restricted to pre-synaptic compartments (Huang et al., 2006). Irrespective of this difference these findings might help to explain how Orb2 might act in synapses. Pre-synaptic localization might suggest Orb2 to contribute in vesicle related processes like fusion to the pre synaptic membrane. By contrast, if Orb2 were primarily post-synaptic, as was shown for mCPEB3, Orb2 targets would probably be involved in receptor modification/shuttling or general modifications of the post synaptic density.

3.3.3 Orb2 has pleiotropic roles in development and memory formation

The generated orb2^{attP} mutants are lethal with only a few escapees, which show a strong locomotion phenotype and die soon after eclosion. This is consistent with the previously published observation that Orb2 in addition to its acute function in long-term memory is also critically required during development (Keleman et al., 2007). To directly test Orb2 function in long-term memory in adults we used FRT flanked rescue constructs to remove Orb2 by co-expression of FLPase. This enabled a tissue specific knock-out of the orb2 gene, thus bypassing the developmental functions of Orb2 by leaving other tissues intact. Using this strategy, we confirmed Orb2 to be required for long-term memory formation in MB γ lobes. Interestingly, removal of Orb2 in MBs also blocks short-term memory. This finding is in line with Orb2 having a role during development and in long-term memory. Further we found that removal of Orb2 from fru+ neurons completely abolishes courtship, again strongly suggesting that Orb2 is involved more generally in neuronal function and neuronal development (Fig.3).

To exclude any developmental defects in the Orb2 depleted neurons as the cause for the observed memory phenotype it will be important to study the developmental role of Orb2 in more detail. It was shown that other CPEB proteins could influence dendritic branch size and number (Bestman and Cline,

2008, 2009). It will be interesting to see whether Orb2 also has such a role and whether this function is used during development and/or in acute synapse formation during long-term memory formation. At the level of EM sections homozygous null mutants showed severe damage of the neuronal tissue. Consistently, also light microscopic analysis of homozygous escapees of lethal mutations showed huge variation in signal level when analyzed for the neuropil marker Nc82. It will be important to test by EM whether viable mutants like the Orb2^{ΔQ} or Orb2^{ΔA} do not show such developmental defects, even if they might be subtler than for the knock-out.

3.3.4 Orb2A and Orb2B isoform act in a non-redundant manner in long-term memory and development

Our previous work on Orb2 using the UAS-Gal4 system to rescue the knock-out phenotype, suggested that Orb2A and Orb2B act in a redundant manner with respect to long-term memory (Keleman et al., 2007). We revisited these experiments by generating isoform specific null mutants and found Orb2A and Orb2B to be required for long-term memory and Orb2B having additional roles during development.

Behavioral analyses of isoform specific Q-domain deletion suggest this domain to be required and sufficient in Orb2A and to be dispensable in Orb2B for long-term memory. To unravel the nature of the glutamine stretch we generated Orb2 chimeras in which we replaced the Orb2 Q-domain with homologues of other CPEB proteins and yeast prion proteins. Surprisingly the Q-domains of mCPEB3 and AcCPEB1 could fully rescue the long-term memory defect of Orb2^{ΔQGFP} mutant flies. One suggested mechanism of CPEB function is that neuronal activation leads to a conformational switch of the protein to an aggregated state, which in turn would be a switch to act as translational activator of target RNA (Si et al., 2010). Indeed our swap experiments with homologous Q-rich domains in flies support the idea that this domain might have prion-like properties similar to AcCPEB1. These rescue experiments might also suggest

that another simple mechanism, in which coiled-coil structures mediate aggregation is unlikely to be the case. However, while substitutions with homologous Q-rich domains suggest a prion-like mechanism, it does not exclude different mechanisms like protein-protein interaction. Possible roles for such interaction might involve anchoring of Orb2 to the activated synapse and thereby providing a stable mark of the synapse.

In contrast to the Q-rich domain we found the Orb2 RBD to be required and sufficient for Orb2B function and to be dispensable for Orb2A function in long-term memory. To further define the function of this domain we generated chimeras in which we substituted the endogenous Orb2 RBD by RBD domains of CPEB class I and II. We found that only RBDs of the Orb2/CPEB2-4 subfamily can rescue the lethality phenotype of the orb2 mutants, whereas substitutes with RBDs of the CPEB1 subfamily cannot. This suggests that the CPEB 2-4 subfamily might have different targets than CPEB1 (orb). A recent report on Orb2 identified 24 potential targets (Mastushita-Sakai et al., 2010). Some of these are established Orb1 targets and consistently carry CPE elements in their 3'UTRs. Interestingly, mutating the CPE elements within these 3'UTRs did not significantly change the binding properties of Orb2 (Mastushita-Sakai et al., 2010). Therefore we do think it is unlikely that Orb2 does recognize CPE motifs, although the underlying targets of Orb1 and Orb2 might partially overlap.

Surprisingly, CPEB2-4 RBD domains could not rescue the long-term memory phenotype. This suggests the underlying targets to be highly conserved within CPEB2-4 subfamily for development but not for memory function. This is surprising to some extent since so far most of the identified long-term memory specific genes have been incredibly well conserved throughout different model organisms. However, the RBD might require other parts of the protein for its specificity and it might be these parts that differ between different CPEB proteins. The Q-domain in theory might contribute to this specificity by changing the overall structure of the protein. This might also explain why some homologous Q-rich domains can rescue the long-term memory phenotype and others not.

However, our finding that the Q-domain is required and sufficient in Orb2A, which itself is dispensable of the RBD function argues against such a model.

Irrespective of why CPEB class II RBDs can rescue the development but not the long-term memory function of Orb2, these findings provided us with the great opportunity to dissect these two functions further apart. The long-term memory phenotype of flies, in which the RBD has been replaced by homologous mCPEB classII RBD, could be fully rescued by Orb2B but not Orb2A. This might suggest that in addition to a developmental role of the RBD in Orb2B there is also a requirement for long-term memory formation. However, this result might be due to the mCPEB RBD replacement acting as a hypomorph. We do think this unlikely to be the case because these flies have completely normal development and short-term memory. Nonetheless, using the Gal80 repressor system to dissect the temporal requirements of the RBD could answer the temporal requirements of this domain in more detail.

To define the specific residues required for RNA binding and specificity of CPEB class II we aligned several CPEB proteins and identified conserved residues required to contact the mRNA. When mutated we found RRM1, RRM2 and the Znf motif to be absolutely required for viability, resembling the knock-out phenotype (Fig.6). Experiments in which we substituted the Orb2 RBD with mCPEB2 RBD suggested a functional role of the RBD in Orb2B only. Consistently experiments with mutated RBD suggest that this domain is dispensable in Orb2A.

CLIP experiments suggest RRM1 and RRM2 to be critically required for RNA binding. By contrast, mutation of the Znf does not reduce RNA binding of Orb2. These results could be reconciled if the binding in Znf mutants is, while still present, now unspecific.

3.3.5 Complementation between Orb2 alleles suggests interaction between A and B isoforms

Our results suggested that Orb2A and Orb2B both have a role in long-term memory formation, albeit using different domains to contribute to this process. This suggests that the isoforms act by different mechanism and ultimately poses the question of whether they would function independently from each other or whether they would act in concert. Evidence that the latter might be the case came from complementation experiments in which Orb2A with a nonfunctional RBD crossed to Orb2B devoid of its Q-domain could fully rescue the long-term memory phenotype. However, this can be achieved either by direct interaction or by independent function of the two isoforms.

To differentiate between these two possibilities and because Orb2 might function in a prion-like manner in long-term memory (Heinrich and Lindquist, 2011; Si et al., 2010), we biochemically analyzed aggregation behavior of Orb2. First, we overexpressed orb2 constructs in S2 cells. At the level of light microscopy Orb2A had a punctuate appearance, which was fully dependent on its Q-domain. Immunoprecipitation experiments showed high molecular weight oligomeres to be dependent on the N-terminal Q-domain. On the other hand, Orb2B did not show punctuate structures, yet immunoprecipitation experiments did show high molecular weight oligomeres. As with Orb2A these oligomers were also dependent on the Q-rich domain. It is important to note that the experiments in S2 cells involve overexpression of the constructs, which can often lead to non-physiological aggregates. In contrast to experiments done by Si et al., 2010 we did not use serotonin or any other neurotransmitter to trigger aggregation. Since S2 cells are not of neuronal origin it is an open question how relevant the S2 experiments are to a potential role for Orb2 complexes in memory.

Importantly, *in vivo* we also observed high molecular weight complexes when both isoforms are present. To test whether the complementation we observed in behavioral assays stem from a potential interaction of the two isoforms, we performed biochemical analysis of isoform specific mutants. In contrast to experiments in S2 cells *in vivo* Orb2B has a significantly reduced

ability to form oligomers in case where Orb2A is lacking, while most of the Orb2A protein seems to exist in the oligomeric conformation. This suggested that Orb2B is able to form oligomers *in vivo*, but this oligomerization requires Orb2A to be present.

Surprisingly, when we looked for aggregates in Q-deletion mutants we did not find any decrease in oligomerization of Orb2. This is in contrast to our finding in S2 cells where removal of Orb2A N-terminus abolished oligomer formation. However, these experiments tested Q-domain deletion of both isoforms simultaneously. Since our behavioral data suggests that the Q-domain is required and sufficient for Orb2A function but not required for Orb2B function it will be important to specifically test Orb2A lacking the Q-domain. Nonetheless, our finding that Q-domain mutants are still able to form complexes strongly suggests additional proteins to be involved in oligomer formation. Evidence that additional proteins might be involved stems from our observation that so far we failed to co-immunoprecipitate Orb2A and Orb2B, both *in vivo* and *in vitro* (data not shown). This argues against a model in which Orb2A and Orb2B directly interact with each other. By contrast, mass spec analysis of the Orb2 oligomeric bands suggests couple proteins, involved in cytoskeleton stability and translational control to be part of this complex (data not shown).

One question that needs to be solved is whether the observed oligomers are relevant for long-term memory formation. If so one should be able to observe an increase in oligomers after learning. Collecting enough brains from behavioral assays is a near impossible experiment. But one could indirectly test this by activating MB neurons with the temperature sensitive channel TRPA1 and subsequently looking for an increase in aggregate formation.

3.3.6 The Orb2 3'UTR is required for long-term memory formation in an isoform specific way

Beside CPEB many other RNA binding proteins have been implicated in the process of long-term potentiation and memory (Mayford et al., 1996; Mori et al.,

2000; Rehbein et al., 2000; Tiruchinapalli et al., 2003). Are they all in independent pathways controlling their specific sets of RNA targets or are they interconnected? To test if Orb2 itself is a subject to such a translational regulation, likely through its 3'UTR, we tested this by substitution of its endogenous 3'UTR with the SV40 3'UTR. Indeed we found that the 3'UTR is critically required for the function of both isoforms. Beside the behavioral affect on courtship and long-term memory, we find additional indications for a strong post- transcriptional regulation based on the finding that Orb2B is heavily upregulated upon mutation of its 3'UTR (Fig.8). As a single miRNA, mir-8, is predicted to function in the 3' UTR of Orb2 we generated mutations for this site and will soon be able to test these flies.

Interestingly in addition to the miRNA-binding site a recent report by Mastushita-Sakai et al., 2010 suggested Orb2 binding sites in its own 3'UTR. This strongly suggests a feed back loop whereby Orb2 might control its own translation.

3.4 Working model for Orb2 function in long-term memory

Frey and Morris proposed the model of a synaptic tag to explain the synapse specific strengthening that is thought to underlie memory (Frey and Morris, 1997). Further work from the Kandel lab elaborated this model to suggest a synaptic tag consisting of multiple components (Casadio et al., 1999; Miniaci et al., 2008). In their model a first period of synaptic activity would lead to a marking process, which is transcription and translation independent. A later, sustained phase of the tagging process would require transcription and translation to keep the potentiation of the synapse beyond 48hrs (Miniaci et al., 2008). The first component of the tag would very quickly modify the synapse in a way that improves and strengthens the synaptic signaling. The second part of the synaptic tag might then maintain these modifications over a longer period of time. This second part would require translation of new proteins locally at these synapses.

In this section I would like to try to fit our findings into this existing model and propose that Orb2 might fulfill the requirements for both parts of the synaptic tag. First, we showed that Orb2 is acutely required during the time of training but is dispensable for memory retrieval (Keleman et al., 2007). However this early experiments have been done in a heterozygous background of Orb2^Δ over Orb2^{ΔQ} to overcome the lethality of orb2 null mutations. Thus in all these cases a functional RBD and N-terminus of both isoforms was available all the time. As we have demonstrated in the course of this work this can lead to complementation of the two Orb2 isoforms. Thus we have to reconcile our suggestion that Orb2 is exclusively required during the time of training but not for maintenance of memory. Rather these experiments suggest that a functional Q-rich domain is vital during the time of training for long-term memory. Since the Q-rich domain is sufficient and the RBD is dispensable for Orb2A function it suggests that also Orb2A might be sufficient during the time of training. In addition we found this isoform to exist preliminary in an oligomeric form in adult brains. Thus Orb2A complex formation might serve as an initial tag to mark the synapse for protein synthesis dependent modifications over a long period of time.

The second step of tagging requires translation of new mRNA. In this process mRNA binding proteins have been postulated to play a major role (Si et al., 2003a). While we don't have any direct evidence for such a tag, there are several observations that we made as a result of our behavioral and biochemical experiments that might suggest a role for Orb2B in this step. First, behaviorally we found the Orb2B RBD to be critically required for long-term memory. Although a temporal analysis for the requirements of the RBD still needs to be done, we envision it to be required for the maintenance of the long-term memory in addition to its function during development. In immunoprecipitation experiments from orb2^{ΔA} flies we found reduced amounts of oligomers in comparison to wild type flies. Thus we conclude that Orb2B also can form oligomeres but requires initial oligomerization of Orb2A to do so. Altogether this suggests Orb2B to transport mRNA to a complex initially formed by Orb2A. Orb2B might enter this

complex by other means than its Q-domain, since behaviorally this domain is dispensable for Orb2B function and biochemically removal does not lead to observable defects in oligomerization.

Perhaps these two steps might correspond to the two tags envisioned by the Kandel group. However, at this point my experiments showing these two stages of oligomerization are preliminary at best. In addition, the idea that they correspond to the two steps of synaptic tagging is highly speculative.

A model with two different phases of the synaptic tag would predict the existence of two different populations of Orb2 proteins. One that is already present at or close to the synapse prior activation, required for the first step of tagging and a second, which provides the synapse in a later step with new mRNA required for modification of the synapse. The first might be at least in part locally translated at the synapse. Evidence that this might be the case comes from the observation that both Orb2 isoforms are highly controlled by their 3'UTR. In theory the 3'UTR could be used to control local translation of Orb2A specifically at or close to synapses, which in turn might explain its low abundance. However, it will be important to localize the Orb2A protein to make further predictions on its function in long-term memory.

The second population of Orb2 would be predicted to be present in the cell body to bind target mRNAs to transport them towards the synapse (possibly via RNP mediated transport). Our finding that Orb2B is present in RNP structures, which are thought to mediate RNA transport, supports this idea. The finding that it is actually Orb2B that requires a functional RBD further supports this idea. By visualizing the dynamics of both the Orb2 mRNA using MS2 tagged RNA and the Orb2 protein using photoactivatable tags should we should be able to further address this question.

In summary we have shown that both Orb2A and Orb2B are critically required for long-term memory, albeit the isoforms achieve this by different mechanisms. While Orb2A critically requires its Q-rich domain but not its RBD, excluding a function in local translation of mRNA, Orb2B requires its RBD suggesting this isoform to actually mediate local translation.

3.5 Supplementary information

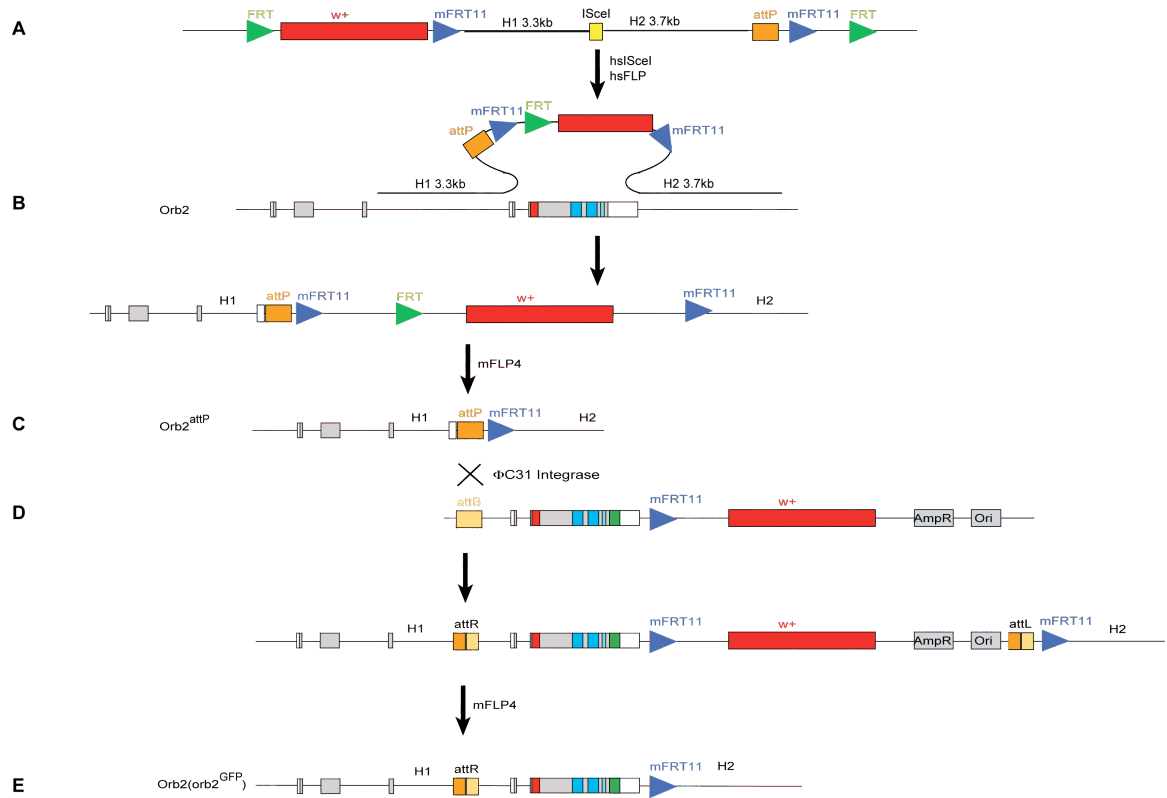


Fig.S1 Targeting and rescue scheme.

(A, B) Linearisation and excision of the donor element using hslFLP and hslScel resulted in direct replacement of the region between the two homologous arms by the vector inserts (attP, mFRT11, FRT, W+, mFRT11) (C) The altered specificity FLP recombinase mhsFLP4 was used to precisely excise the *w+* marker, flanked by two mFRT11 sites to obtain the final mutant fly (D) site directed integration of the attB construct into *orb2*^{attP}, using ΦC31 integrase. (E) mhsFLP4 was used to precisely excise the *w+* marker, flanked by two mFRT11 sites, to obtain the final rescue fly.

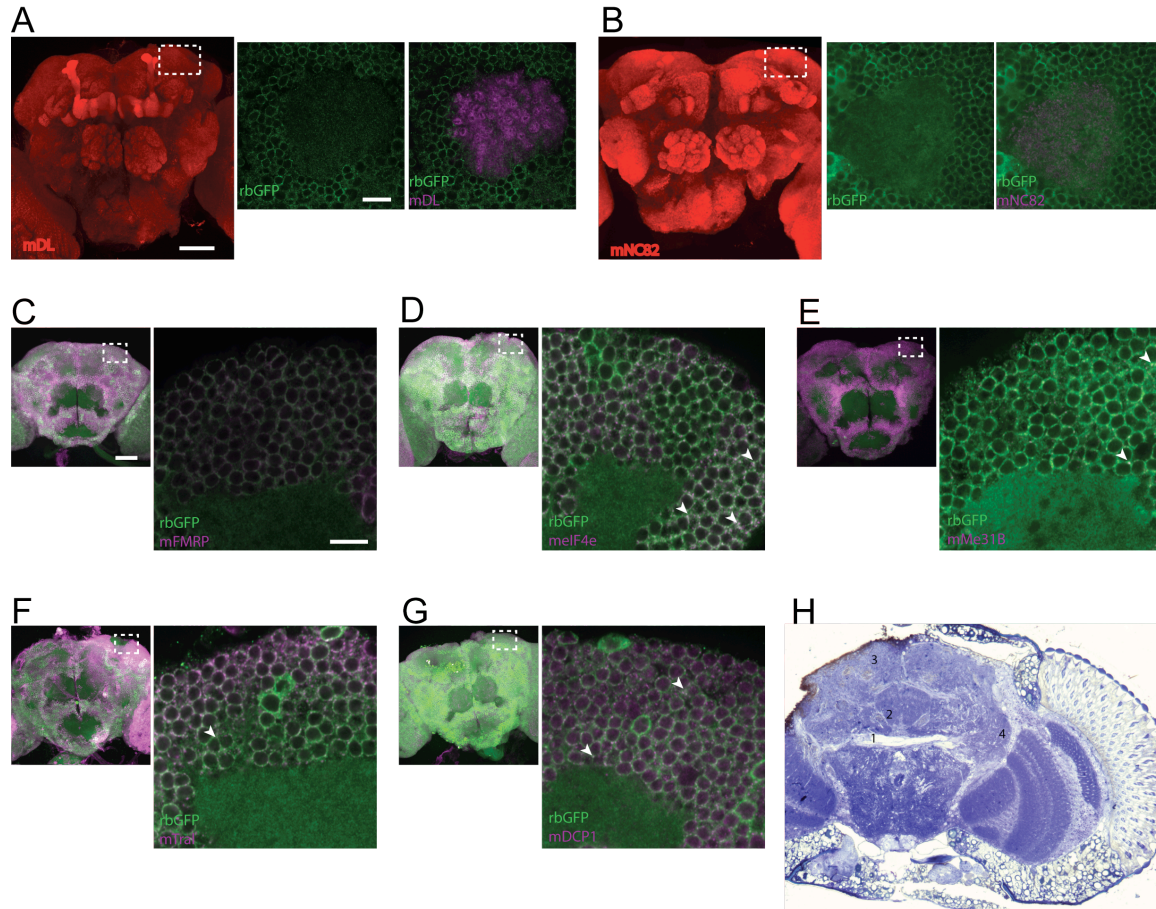


Fig.S2 Orb2 co-localization studies.

Orb2^{GFP} counterstained with (A) the post-synaptic marker DiscLarge (B) the pre-synaptic marker NC82. Neither the DL nor Nc82 show overlap with Orb2, despite Orb2 being present in similar areas. (C-G) Orb2^{GFP} counterstained with RNP antibody marker (C) FMRP (D) EIF4e (E) Me31B (F) Tral (G) DCP1 (H) Toluenblue staining of adult brain slices to localize the MB calyx for immuno EM Scale bar: 50μm

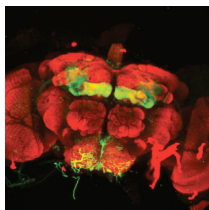


Fig.S3 Immunofluorescence staining of VT29317G4.

VT29317G4 driving a UAS-cd8:GFP construct, stained with αGFP and αNC82 shows the driver line to be specifically expressed in mushroom body gamma lobe

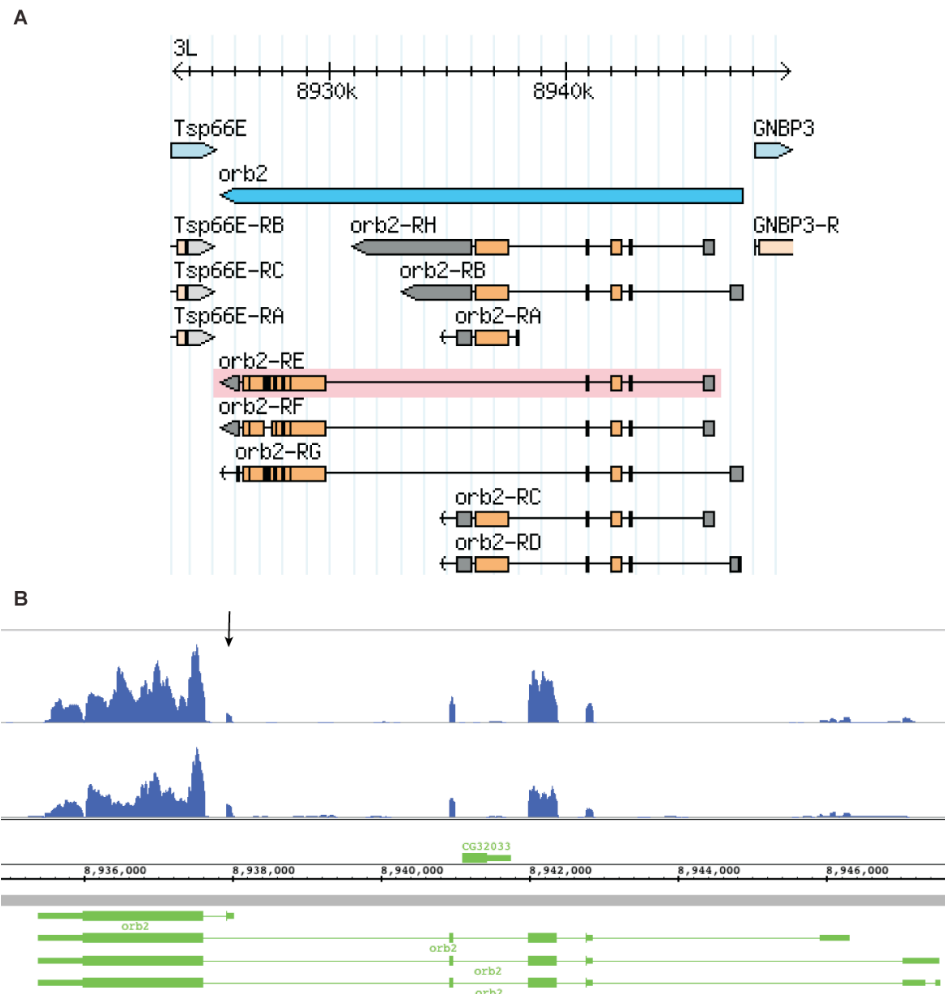


Fig.S4 Organisation of the orb2 genomic locus.

(A) The orb2 locus can potentially express 8 different isoforms (according to deep sequencing data in Flybase) (B) deep sequencing data from our lab showing the orb2A exon to be expressed at very low levels. Arrowhead indicates Orb2A exon.

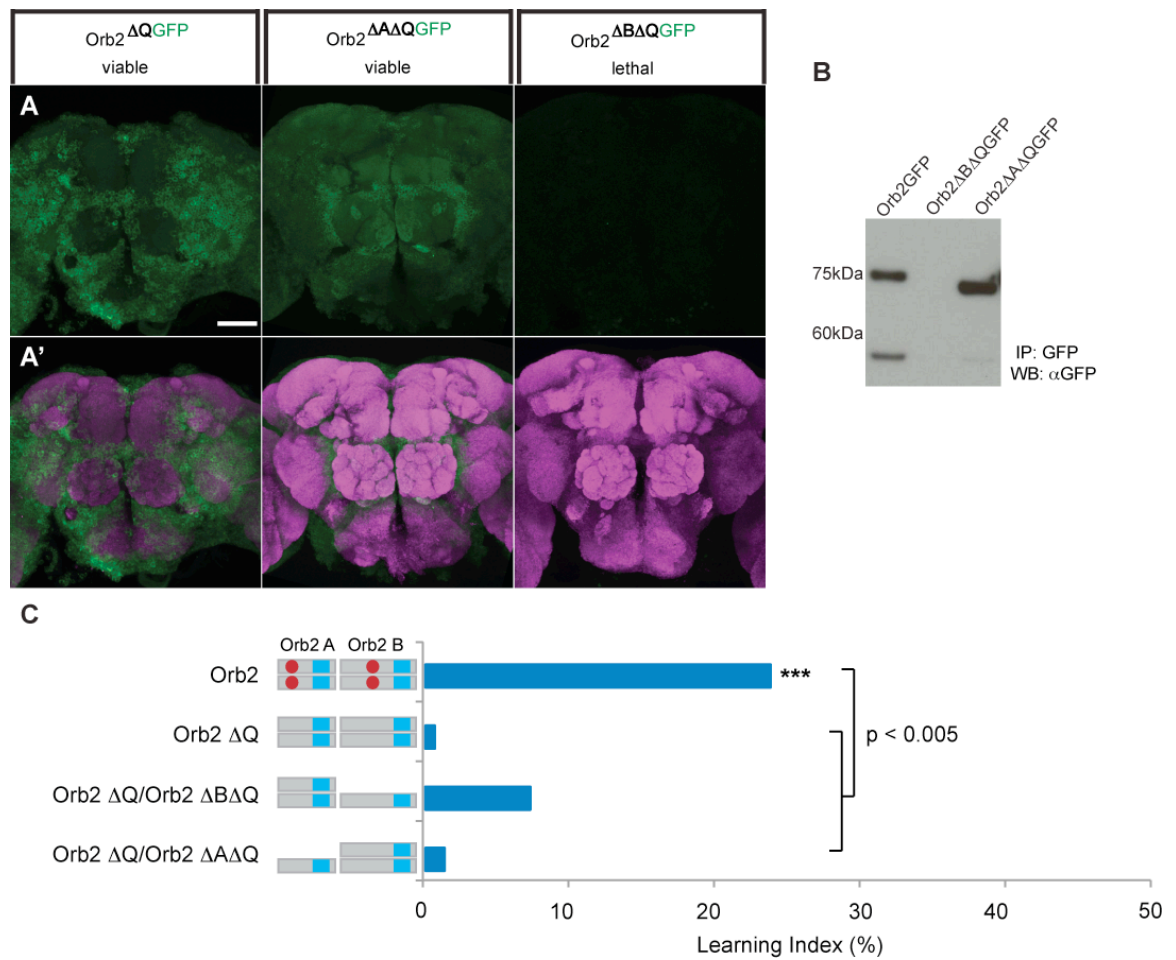


Fig.S5 Isoform specific deletion of the Q-domain.

(A) immunostaining of GFP tagged isoform specific Q-domain deletion using α GFP and (A') α GFP and α NC82 (B) IP experiment shows no altered expression level in comparison to WT (C) LTM assay of GFP tagged isoform specific Q-domain deletion. P values are for H_0 : $LI = LI_{Orb2GFP}$ (permutation test) and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for H_0 , $LI = 0$ (permutation test)

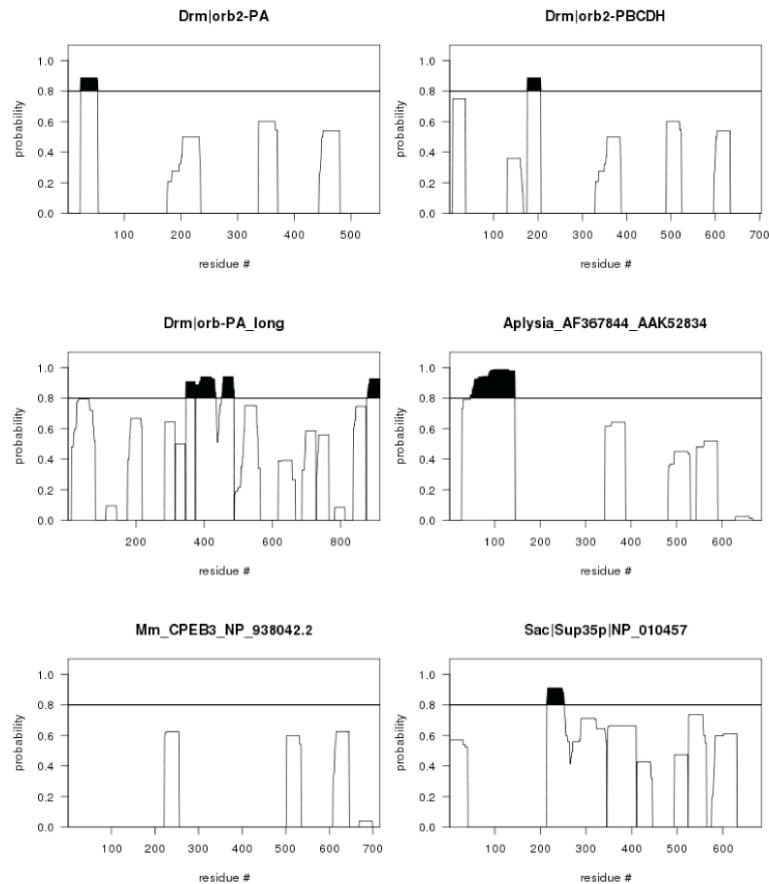


Fig.S6 Coiled-coil prediction. Probability to form coiled-coils of proteins used to replace the Orb2 Q-domain. Probability was calculated as 1 - p-score as described in (Fiumara et al., 2011)

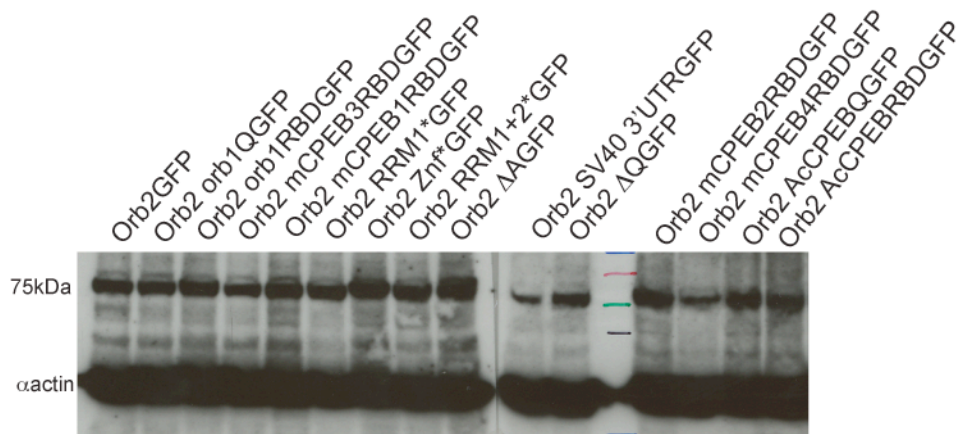


Fig.S7 Western Blot verification of rescue alleles probed with αGFP and αactin used as loading control

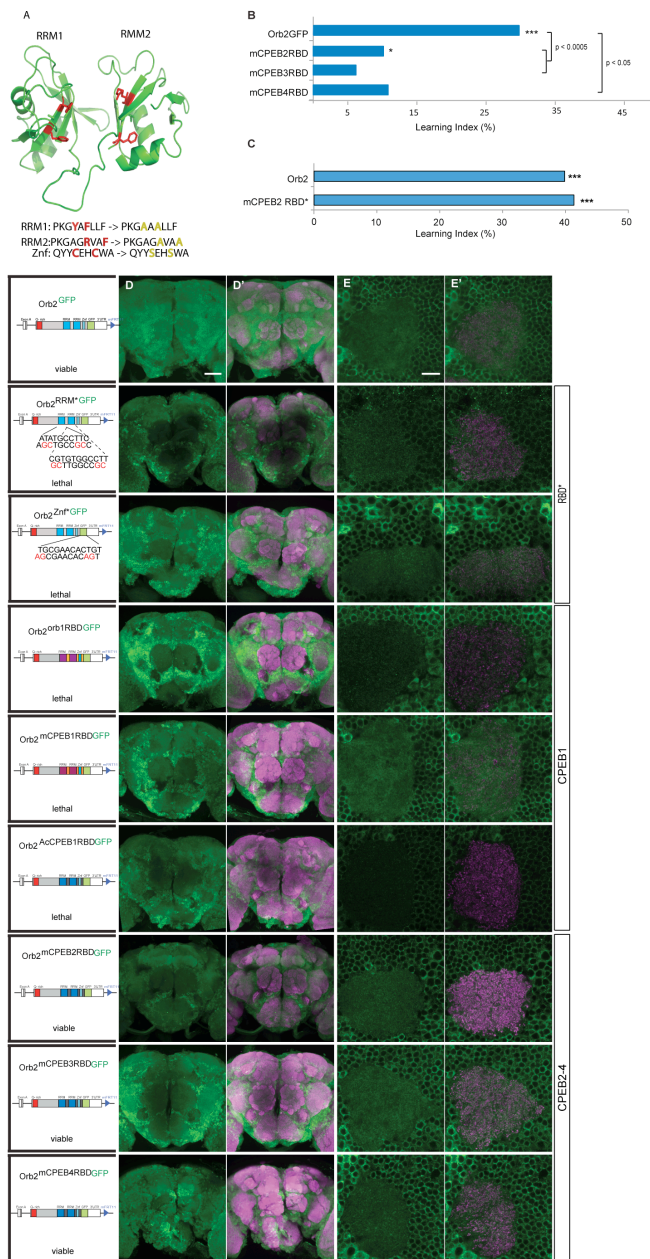


Fig.S8 Modifications of the endogenous Orb2 RBD.

(A) model of Orb2 RBD. Red residues are predicted to contact mRNA and were specifically mutated. (B) LTM assay of RBD substitutions with mCPEB class II RBDs (C) STM assay of RBD substitutions with mCPEB class II RBDs. P values are for H_0 : $LI = LI_{Orb2GFP}$ (permutation test) and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for H_0 , $LI = 0$ (permutation test) (D) immunofluorescence staining of adult heads using α GFP and (D') α GFP and α NC82 (E) immunofluorescence staining of the mushroom body using α GFP and (E') α GFP and α NC82

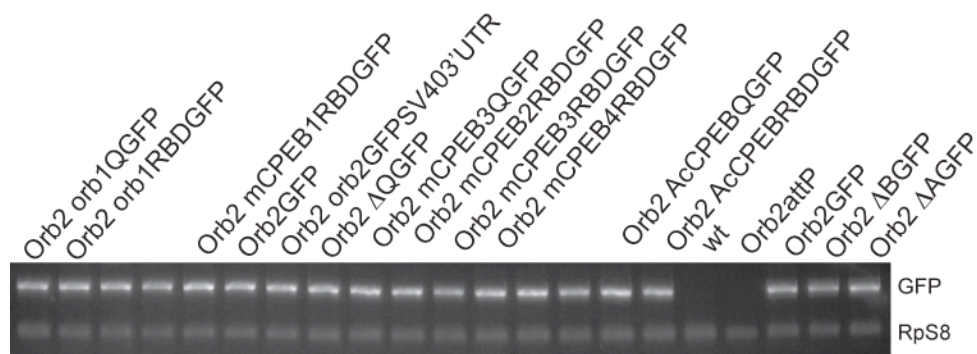


Fig.S9 RT verification of rescue alleles. Primers against the GFP tag of each construct have been used for verification. RpS8: ribosomal protein used as internal reference control

Genotype	homozygous flies (%)	n	SEM
Orb2GFP	36.62	442	0.016
orb2attP	2.41	375	0.008
Orb2ΔB	1.3	332	0.008
Orb2ΔA	27.03	380	0.017
Orb2ΔQ	28.05	371	0.041
Orb2ΔAΔQ	25.82	376	0.045
Orb2ΔBΔQ	0.86	336	0.004
Orb1Q	24.27	407	0.081
AcQ	30.41	428	0.041
mCPEB3Q	28.52	540	0.018
sup35Q	39.01	381	0.035
Ure2Q	35.08	573	0.035
RRM1*	0.67	314	0.006
RRM2*	1.34	370	0.006
Znf*	6.04	365	0.016
RRM1+2*	2.85	223	0.014
Orb2ΔARRM1+2*	0.97	332	0.005
Orb2ΔBRRM1+2*	4.12	333	0.005
Orb1RBD	4.48	319	0.044
AcCPEB1 RBD	3.93	341	0.007
mCPEB1RBD	1.32	323	0.008
mCPEB2RBD	27.82	324	0.005
mCPEB3RBD	27.64	509	0.022
mCPEB4RBD	30.31	715	0.022
Orb2 SV40	29.76	469	0.047
Orb2ΔA SV40	19.58	392	0.028
Orb2 ΔB SV40	0.6	341	0.003

ST1 Viability of rescue alleles. Percentage of homozygous viable flies in a TM3Sb balanced stock± SEM.

Genotype	CI- (%)	n	CI+ (%)	n	LI (%)	LI=0	P (LI=LI _{orb2GFP})
Wt ^{iso}	80.25±0.02	101	58.41±0.02	107	32.56	<0.0001	0.883
Orb2 ^{ΔQGFP} /orb2 ^{attP}	74.92±0.03	60	69.56±0.03	57	7.14	0.124	0.00028
Orb2 ^{ΔQGFP}	69.31±0.02	101	68.37±0.02	92	1.35	0.388	<0.0001
Orb2 ^{GFP}	79.81±0.02	106	54.49±0.02	108	31.72	<0.0001	

ST2 LTM assay of Orb2^{attP}. Courtship indices of naive (CI-) and experienced (CI+) males of the indicated genotype in single-pair assays with mated females as trainers and testers, shown as $CI_{mean} \pm SEM$ of n males. P values for the null hypothesis that naive and experienced males court equally (LI=0) or for the null hypothesis that mutant and corresponding control males learn equally well (LI=LI_{Orb2GFP}), both p values are calculated by permutation test (see Fig.1).

Genotype	CI- (%)	n	CI+ (%)	n	LI (%)	LI=0	P (LI=LI _{orb2GFP})
Wt ^{iso}	47.38±1.7	93	34.96±1.2	96	26.2	<0.0001	0.263
Orb2 ^{ΔQGFP} /orb2 ^{attP}	46.28±2.8	53	33.71±2.8	49	27.16	0.0006	0.916
Orb2 ^{ΔQGFP}	35.79±1.3	47	22.76±1.5	51	36.38	<0.0001	0.232
Orb2 ^{GFP}	47.38±1.7	93	34.96±1.2	96	26.2	<0.0001	
Orb2 ^{ΔA}	49.78±1.9	54	39.81±1.4	54	20.04	<0.0001	0.287

ST3 STM assay of Orb2^{attP}. Courtship indices of naive (CI-) and experienced (CI+) males of the indicated genotype in single-pair assays with mated females as trainers and testers, shown as $CI_{mean} \pm SEM$ of n males. P values for the null hypothesis that naive and experienced males court equally (LI=0) or for the null hypothesis that mutant and corresponding control males learn equally well (LI=LI_{Orb2GFP}), both p values calculated by permutation test (see Fig.1).

Genotyp	CI- (%)	n	CI+ (%)	n	LI (%)	LI=0	P (LI=LI _{orb2GFP})
>>Orb2 ^{GFP} >>, UAS-FLP	49.72±4.2	44	39.60±2.0	46	20.34	0.012	
>>Orb2 ^{GFP} >>, MB247, UAS-FLP	54.11±2.1	42	52.77±2.1	47	2.47	0.329	0.002
>>Orb2 ^{GFP} >>, VTFLPg	44.57±2.0	39	40.16±2.0	49	9.88	0.067	0.135
Orb2 ^{GFP} , >>Orb2 ^{GFP} >>, VTFLPg	45.816±2.3	42	38.419±2.2	49	16.23	0.013	0.611

ST4 LTM assay for conditional alleles. Courtship indices of naive (CI-) and experienced (CI+) males of the indicated genotype in single-pair assays with mated females as trainers and testers, shown as $CI_{mean} \pm SEM$ of n males. P values for the null hypothesis that naive and experienced males court equally (LI=0) or for the null hypothesis that mutant and corresponding control males learn equally well (LI=LI_{>>Orb2GFP>>, UAS-FLP}), both p values calculated by permutation test (see Fig.3).

Genotype	CI- (%)	n	CI+ (%)	n	LI (%)	LI=0	P (LI=LI _{orb2GFP})
>>Orb2 ^{GFP} >>, UAS-FLP	43.18±1.9	45	31.90±2.2	53	26.11	<0.0001	
>>Orb2 ^{GFP} >>, VTFLPg	47.27±2.1	70	44.219±2.0	72	6.45	0.117	0.0005
Orb2 ^{GFP} , >>Orb2 ^{GFP} >>, VTFLPg	47.291±2.1	54	37.062±2.1	54	21.63	0.0004	0.555
>>Orb2 ^{GFP} >>, FruFLP	14.9±0.01	30					
>>Orb2 ^{GFP} >>, FruFLP	20.94±0.02	29					

ST5 STM assay for conditional alleles. Courtship indices of naive (CI-) and experienced (CI+) males of the indicated genotype in single-pair assays with mated females as trainers and testers, shown as $CI_{mean} \pm SEM$ of n males. P values for the null hypothesis that naive and experienced males court equally (LI=0) or for the null hypothesis that mutant and corresponding control males learn equally well (LI=LI_{>>Orb2GFP>>, UAS-FLP}), both p values calculated by permutation test (see Fig.3).

Genotype	CI- (%)	n	CI+ (%)	n	LI (%)	LI=0	P (LI=LI _{orb2GFP})
Orb2 ^{GFP}	80.25±0.02	101	58.41±0.02	107	27.21	<0.0001	
Orb2 ^{ΔQ}	69.31±0.02	101	68.37±0.02	92	1.35	0.388	<0.0001
Orb2 ^{ΔQ} /Orb2 ^{ΔB}	79.33±0.02	89	54.27±0.02	89	31.58	<0.0001	0.46
Orb2 ^{ΔQ} /Orb2 ^{ΔA}	78.27±0.02	101	68.3±0.02	94	12.73	0.0003	0.0005
Orb2 ^{ΔA}	78.67±0.02	83	69.17±0.02	78	12.08	0.001	0.0018
Orb2ΔAΔQ	71.25±0.03	72	69.70±0.03	67	2.17	0.367	0.0007
Orb2ΔA/Orb2ΔB	82.76±0.02	58	67.54±0.02	69	18.39	<0.0001	0.299
Orb2ΔA/Orb2ΔBΔQ	79.23±0.02	52	76.96±0.02	51	2.86	0.275	<0.0001
Orb2ΔB/Orb2ΔAΔQ	82.64±0.02	53	68.62±0.03	47	16.97	0.001	0.277

ST6 LTM assay of isoform specific alleles. Courtship indices of naive (CI-) and experienced (CI+) males of the indicated genotype in single-pair assays with mated females as trainers and testers, shown as $CI_{mean} \pm SEM$ of n males. P values for the null hypothesis that naive and experienced males court equally (LI=0) or for the null hypothesis that mutant and corresponding control males learn equally well (LI=LI_{Orb2GFP}), both p values calculated by permutation test (see Fig.4).

Genotype	CI- (%)	n	CI+ (%)	n	LI (%)	LI=0	P (LI=LI _{orb2GFP})
Orb2 ^{GFP}	77.63±0.02	65	59.18±0.02	61	23.75	<0.0001	
Orb2 ^{ΔQGFP}	69.31±0.02	101	68.37±0.02	92	1.35	0.388	<0.0001
ΔQ/Orb2ΔBΔQ	81.05±0.02	57	75.18±0.02	57	7.25	0.053	0.0004
ΔQ/Orb2ΔAΔQ	70.87±0.03	52	69.90±0.03	51	1.35	0.418	0.001

ST7 LTM assay of isoform specific alleles with deleted Q-domain. Courtship indices of naive (CI-) and experienced (CI+) males of the indicated genotype in single-pair assays with mated females as trainers and testers, shown as $CI_{mean} \pm SEM$ of n males. P values for the null

hypothesis that naive and experienced males court equally (LI=0) or for the null hypothesis that mutant and corresponding control males learn equally well (LI=LI_{Orb2GFP}), both p values calculated by permutation test (see Fig.S5).

Genotype	CI- (%)	n	CI+ (%)	n	LI (%)	LI=0	P (LI=LI _{Orb2GFP})
Orb2 ^{GFP}	46.893±1.4	95	36.046±1.3	99	23.13	<0.0001	
Orb2 ^{ΔQGFP}	31.86±0.9	82	32.35±1.5	78	-1.55	0.613	0.002
orb1Q	46.63±1.5	71	42.36±1.6	65	9.16	0.028	0.172
AcCPEB1Q	47.55±1.2	102	38.09±1.6	87	19.89	<0.0001	0.265
mCPEB3Q	39.53±3.1	22	31.68±3.1	23	19.86	0.04	0.763
sup35	44.33±1.6	60	39.84±1.5	68	10.23	0.023	0.287

ST8 LTM assay of alleles with Q-domain replacements. Courtship indices of naive (CI-) and experienced (CI+) males of the indicated genotype in single-pair assays with mated females as trainers and testers, shown as CI_{mean} ± SEM of *n* males. *P* values for the null hypothesis that naive and experienced males court equally (LI=0) or for the null hypothesis that mutant and corresponding control males learn equally well (LI=LI_{Orb2GFP}), both p values calculated by permutation test (see Fig.5).

Genotype	CI- (%)	n	CI+ (%)	n	LI (%)	LI=0	P (LI=LI _{Orb2GFP})
Orb2 ^{GFP}	48.64±2.2	65	43.22±1.3	68	29.65	<0.0001	
mCPEB2RBD	38.30±1.1	102	34.76±1.08	108	9.25	0.012	<0.0001
mCPEB3RBD	38.84±1.6	72	36.63±1.8	65	5.69	0.181	0.0003
mCPEB4RBD	34.50±1.8	75	31.09±1.6	78	9.87	0.088	0.01

ST9 LTM assay of alleles with mCPEB2-4 RBD replacements. Courtship indices of naive (CI-) and experienced (CI+) males of the indicated genotype in single-pair assays with mated females as trainers and testers, shown as CI_{mean} ± SEM of *n* males. *P* values for the null hypothesis that naive and experienced males court equally (LI=0) or for the null hypothesis that mutant and corresponding control males learn equally well (LI=LI_{Orb2GFP}), both p values calculated by permutation test (see Fig.S8).

Genotype	CI- (%)	n	CI+ (%)	n	LI (%)	LI=0	P (LI=LI _{Orb2GFP})
Orb2 ^{GFP}	77.55±0.02	94	55.00±0.02	95	29.08	<0.0001	
mCPEB2RBD	60.72±0.02	69	55.83±0.02	72	8.05	0.115	0.003
mCPEB2RBD/Orb2ΔA	80.87±0.02	63	58.77±0.02	65	27.33	<0.0001	0.784
mCPEB2RBD/Orb2ΔB	25.51±0.03	35	27.98±0.03	36	-9.69	0.699	0.034

ST10 LTM assay of alleles with mCPEB2 RBD replacement. Courtship indices of naive (CI-) and experienced (CI+) males of the indicated genotype in single-pair assays with mated females as trainers and testers, shown as $CI_{\text{mean}} \pm \text{SEM}$ of n males. P values for the null hypothesis that naive and experienced males court equally (LI=0) or for the null hypothesis that mutant and corresponding control males learn equally well (LI=LI_{Orb2GFP}), both p values calculated by permutation test (see Fig.6A).

Genotype	CI- (%)	n	CI+ (%)	n	LI (%)	LI=0	P (LI=LI _{Orb2GFP})
Orb2 ^{GFP}	84.30±0.02	50	48.60±0.02	50	40	<0.0001	
mCPEB2RBD	78.85±0.02	52	47.31±0.02	52	42.34	<0.0001	0.771

ST11 STM assay of alleles with mCPEB2 RBD replacement Courtship indices of naive (CI-) and experienced (CI+) males of the indicated genotype in single-pair assays with mated females as trainers and testers, shown as $CI_{\text{mean}} \pm \text{SEM}$ of n males. P values for the null hypothesis that naive and experienced males court equally (LI=0) or for the null hypothesis that mutant and corresponding control males learn equally well (LI=LI_{Orb2GFP}), both p values calculated by permutation test (see Fig.S8).

Genotyp	CI- (%)	n	CI+ (%)	n	LI (%)	LI=0	P (LI=LI _{Orb2GFP})
Orb2 ^{GFP}	77.63±0.02	65	59.18±0.02	61	23.75	<0.0001	
ΔQ/RBD*	80.32±0.02	63	59.85±0.02	67	25.48	<0.0001	0.758
Orb2ΔA/RBD*	90.01±0.01	46	74.51±0.02	51	17.31	<0.0001	0.125
Orb2ΔA/Orb2ΔB	82.76±0.02	58	67.54±0.02	69	18.39	<0.0001	0.299
Orb2ΔA/Orb2ΔB RBD*	83.13±0.02	64	68.19±0.02	69	17.96	0.0001	0.155
Orb2ΔAΔQ/Orb2ΔB RBD*	77.13±0.03	54	65.56±0.03	54	15	0.005	0.173

ST12 LTM assay of RBD* alleles. Courtship indices of naive (CI-) and experienced (CI+) males of the indicated genotype in single-pair assays with mated females as trainers and testers, shown as $CI_{\text{mean}} \pm \text{SEM}$ of n males. P values for the null hypothesis that naive and experienced males court equally (LI=0) or for the null hypothesis that mutant and corresponding control males learn equally well (LI=LI_{Orb2GFP}), both p values calculated by permutation test (see Fig.6B).

Genotype	CI- (%)	n	CI+ (%)	n	LI (%)	LI=0	P (LI=LI _{orb2GFP})
Orb2 ^{GFP}	77.55±0.02	94	55.00±0.02	95	29.08	<0.0001	
SV40 3'UTR	28.63±1.2	99	28.63±1.1	120	0	0.501	<0.0001
Orb2ΔA/Orb2ΔB SV40 3'UTR	46.15±1.3	73	47.55±2.6	58	-3.09	0.693	<0.0001
Orb2ΔB/ Orb2ΔA SV40 3'UTR	32.77±1.0	83	33.19±1.3	89	-1.28	0.589	<0.0001

ST13 LTM assay of alleles with replaced 3'UTR. Courtship indices of naive (CI-) and experienced (CI+) males of the indicated genotype in single-pair assays with mated females as trainers and testers, shown as $CI_{\text{mean}} \pm \text{SEM}$ of n males. P values for the null hypothesis that naive and experienced males court equally (LI=0) or for the null hypothesis that mutant and corresponding control males learn equally well (LI=LI_{Orb2GFP}), both p values calculated by permutation test (see Fig.8).

b GGAACCTTAAGGACAGCCCCTGCACA
c GACTATTTTGTGTGCGCTCGCTTTTCG
d ATGGCTAGCATACAAATGTCAGACA
SK001 GTTGAGATTGAGATTCGGCAGGCTAGCGCAACTGCAGGAAGAAGGGACAAC
SK002 GTTGTCCCTTCTTCCTGCAGTTGCGCTAGCCTGCCGAATCTCAATCTCAAC
SK003 GCGCGCGCGGCTAGCTCTGTAAATAGCAACAAAATTCCACATC
SK004
CTATGTGATCCTCCGCTTTCCCGAAGTTGTTGGCTACCCAAATTGGCAGATATC
SK006 GCGCGGTACCGATCGCCTGATGCTGTTGACGCTGTATTC
SK007 GCGCGCGCGGCTAGCATGGACAAAAGCAAAACCCAGCCCCAG
SK010 GCGCGGTACCGCTCCAGCGGAACGGGACGTGCCGGG
SK013
GGAAATGGAGGAAACGTCGGCGATGGCGTGGAACGCTACTCTAGAAAGGTGTTTG
SK015
GGAAATGGAGGAAACGTCGGCGATGGCATGCTCAACTATTCTCCCAAGGTCTTCC
SK016 CTATGTGATCCTCCGCTTTCCCGAAGCTCGGGCTGGAGCTGTTGCTGC
SK026 GCGCGCGCGGCTAGCTCGGATTCAAACCAAGGCAACAATCAGC
SK027 TATGTGATCCTCCGCTTTCCCGAAGCGGTTGGGCTTGAGCATTGTAAGCTT
SK033 GGAAATGGAGGAAACGTCGGCGATGGCAACCCCATCTACTCATGCAAGG

SK034 CGCGGTACCGTTCTTCTGGTTCCTCATTAGGGGGCTATGG
 SK035 GCGCGCGCGGCTAGCGCTTTCTCTCTGGAAGAAGCAGCAGG
 SK036 GCGGCTAGCTGTTCCCAGTCTCAATCACAGCAACAGC
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 SK057 GCGCGAGATCTTAAGGATCTTTGTGAAGGAACCTTACTTCTGTGG
 SK058 CGCGCACTAGTGATCCAGACATGATAAGATACATTGATGAGTTTGG
 SK062
 GCGCGCGCGGCTAGCCTTCGGGAAAGCGGAGGATCACATAGCCCCTCGTCGCCGGG
 SK070
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 SK071 CGCGCGGTACCGTTCCAGCGGAAGTGGATCTGACGTGGGCGATCAGCACC
 SK072 ACTTTTCGAGAGAAGCGTTCAATGCCATCGCCGACGTTTCCTCCATTTCC
 SK073 GGAAATGGAGGAAACGTCGGCGATGGCATTGAACGCTTCTCTCGAAAAGT
 SK074 GCGCGCGCGCGCTAGCGGGGATTACGGGTTTGGAGTGCTAGTGC
 SK075 CGCGCGGTACCGTTCCAGCGGAATGAAATATGCCGAGGGCGGTCACC
 SK124 ATGTACAACAAATTTGTTAATTTTC
 SK128 ATGTACAACAAATTTGTTAATTTTC
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 SK130 GCCGGTGGTTTCTTCAATCGAAAAAGTTGC
 SK148
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 SK149
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 SK150
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 SK151
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SK152

GCGCGGAATTCATGATAATCTACACGAATGATGGCAACCCGCTGGGACTGCAG

SK153 CGCGCTCTAGACTACTTCTTCTTTTTGCCCTTGCCCTGTCCACCAGCGACCGG

SW11 GCGCGGTACCAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCC

SW12 GCGCAGATCTTTTGTATAGTTCATCCATGCCATGTGTAATCC

SW14 GCGCGAATTCGTCGTTGCTTTTTGCCTTTCCGTTG

SW15 GCGCACTAGTAAGCCTTTTCGAGAGAGGGTGAGCAG

SW16 GCGCAGATCTTAACGGCGGCGCTGGTAGGC

SW17 GCGCGGTACCACACCAGCGAAAGGGGACCGCAC

ST14 Primers used for cloning of rescue constructs.

3.6 Materials and methods

Generation and verification of mutants and rescues

mutant generation and cloning strategy. To generate the donor construct, a 3.3kb homology region upstream to the A specific exon using the primers 5'- GCGCAGATCTCTAGGGCAGCAGCGGAACTGAGG-3' and 5'- GCATGCGGCCGACCATCATCGAAACGAGAGGTTTG-3', and a second 3.7kb homology region downstream to the common orb2 exon using the primers 5'- GCGCGGTACCTTGAGTGTGCAGGGGCTGTCCTTA-3' and 5'- GCGCACTAGTGCGTAAAATTGAAATTGTTTCCACATCGTTC-3', were PCR amplified from genomic wt flies and cloned into pEO16ED11 (unpublished Dickson B.J) (Supplementary Fig.1A). 9 donor lines were generated using standard P- element mediated transgenesis, carrying the donor element on the 2nd chromosome. A strategy for “ends- out” homologous recombination was followed (Gong and Golic, 2003). Linearisation and excision of the donor element using hsFLP and hslScel resulted in direct replacement of the region between the two homologous arms by the vector inserts of pEO16ED11 (Supplementary Fig.1B). In our case an attP landing site, a mFRT11 site followed by a w⁺ marker and a second mFRT11 site. The altered specificity FLP recombinase mhsFLP4 was used to precisely excise the w⁺ marker, flanked by two mFRT11 sites, to obtain the final mutant fly (Supplementary Fig.1C). Mutants were verified by Southern blot and PCR sequencing of either the deleted region using the primer 5'- GGAACTTAAGGACAGCCCCTGCACA-3' and 5'- GACTATTTTGTGTCGCCTCGCTTTCG-3' or sequencing of the entire locus using the primer 5'-CATTTTCCCATTTCCTCCCATTTCC-3' and 5'- TTAATGGAAGTGCTGGTGCCGTA-3'.

To obtain Orb2^{GFP} rescue construct, GFP has been PCR amplified by the primers SW11/SW12 and cloned Asp718/BglII into the MCS of pSK4 containing attB- MCS- mFRT11- w⁺. This construct (pSW11) was used to clone the PCR

amplified orb2 3' UTR, using the primers SW15/SW16, downstream to the GFP BglII/SpeI and the Orb2 ORF, using the primers SW14/SW17 in frame upstream to GFP EcoRI/Asp718, thus resulting in C- terminal fusion of Orb2 to GFP. We inserted a NheI site into the common exon after the splice site, by single nucleotide exchange through overlap PCR using the primers SK001 and SK002, to allow easier modification of the common exon (=pSK12). To obtain conditional alleles, the cassette containing Orb2, GFP and orb2 3'UTR was subcloned into pSK8 (attB- FRT- MCS- FRT- mFRT11- w+) resulting in a "FLP- out" allele (pSK11FO) or into pSK10 (attB- MCS- FRT-stop-FRT- MCS2- mFRT11- w+) resulting in a "FLP- in" allele (pSK13b). Note that for the "FLP-in" construct the intergenic region and the isoform A specific exon was cloned into MCS before the stop, whereas the common exon was cloned into MCS2 after the stop to ensure premature termination of both isoforms.

Plasmid DNA was injected into embryos from a cross between orb2^{attP} flies and ΦC31 integrase carrying flies (ZHII, unpublished Dickson and Keleman) (Supplementary Fig.1D). DNA injection resulted into site directed integration of the attB construct into orb2^{attP}. mHSFLP4 was used to remove the w+ marker, resulting in the final rescue fly (Supplementary Fig.1E).

The Orb2^{ΔQGFP} allele was generated by PCR amplification of the common orb2 exon with primers SK062 and SW17 and subsequent cloning into pSK12 with NheI/Asp718, deleting AA168-221 of Orb2.

Orb2 RBD domain was defined to start from AA443. To obtain point mutations in RRM1 (AA448- 510) primers CP148 and CP149 were used in overlap PCR to mutate Y492A, F494A, for mutations in RRM2 (AA556- 634) primers CP150 and CP151 were used in overlap PCR to mutate R601A, F604A, for mutation of the Zinc finger domain primers CP152 and CP153 were used in overlap PCR to mutate C669S, C672S.

The replacement of orb2 3'UTR was obtained from PCR amplification of SV40 3'UTR using the primers SK057 and SK058 and subsequent cloning into pSK12 with BglII/SpeI.

The Orb2^{ΔBGFP} allele was generated by overlap of the PCR fragments obtained from amplification of pSK12 using the primers SW14/ SK054 and SK053/SK055 and subsequently replaced the pSK12 EcoRI/NheI fragment. Same combination of primers was used to generate Orb2^{ΔAGFP} but SK055 was replaced by SK001. To obtain isoform specific ΔQ and RBD* mutants the respective mutant construct was subcloned NheI/Asp718 into the respective isoform specific mutant.

Orb1 was PCR amplified from genomic DNA using the primers SK003 and SK006, amplifying the last common exon of orb1, containing the Q- rich domain and the RNA binding domains, from genomic *Drosophila* wild type w1118 DNA. The Q- rich domain was defined as the very N- terminal region of this fragment, and amplified by SK003 and SK004. The RBD domain was defined by the primers SK015 and SK006. All PCR fragments designed to replace endogenous orb2 domains were used in overlap PCR with complementary orb2 fragments and cloned NheI/Asp718 into pSK12.

mCPEB1 was amplified from Riken cDNA library, using the primers SK034 and SK035. The RBD domain was defined by the primers SK033 and SK034.

mCPEB2 was amplified from mouse cDNA library derived from brain tissue using the primers SK070 and SK071. The RBD domain was defined by the primers SK073 and SK071.

mCPEB3 Q rich- domain was amplified from mouse cDNA library derived from brain tissue using the primers SK007 and SK016. The RBD domain was defined by the primers SK013 and SK010.

mCPEB4 was amplified from mouse cDNA library derived from brain tissue using the primers SK074 and SK075. The RBD domain was defined by the primers SK077 and SK075.

AcCPEB1 cDNA was a gift from Kausik Si and amplified using the primers SK036/SK037. The Q- rich domain was defined by the primers SK036 and SK040. The RBD domain was defined by the primers SK043 and SK037.

Sup35 prion domain was amplified from yeast using the primers SK026 and SK027. For primers see **supplementary Table14**.

Southern Blot. A 1047bp probe was generated by PCR using the primer TGATCGCCTCGAAAGCCAATCCTAC and TTGAAAATCGGACAGCCAAATGCTC. 15µg genomic DNA was digested using EcoRI/ SpeI for 5hrs and heat inactivated at 65⁰C. DNA was precipitated with NaAc, 3M, pH5.2, 1/10 vol and 2.5 vol of 100% ethanol, washed with 70% ethanol and resuspended in 30µl TE. DNA was run on a 0.5% agarose gel at 60V at 4⁰C over night. Gel was then depurinated in 0.2M HCl for 30 min, denaturated (1.5M NaCl, 0.5M NaOH) for 30 min and neutralized (1.5M NaCl, 0.5M Tris pH7.5) for 30min. The gel was blotted in 20x SSC over night. After crosslinking, the membrane was incubated in hybridisation solution (ULTRAhyb® Ultrasensitive Hybridization Buffer, Ambion, AM8670) for 2 hrs before incubation with the labeled probe (Prime-It® Random Primer Labeling Kit, Strategene, 300385) for 16hrs.

RT PCR. All alleles were verified for GFP expression in their transcript using primers SW11 and SW12 (**supplementary Fig8**). For RT PCR total RNA was extracted using Trizol and reverse transcribed using random primers. 25 cycles were used in RT for amplification. RpS8, amplified with primers HH142 and HH143, was used as internal control. As negative control a RT minus reaction was performed. For isoform specific rescues, primers SK128 and SK129 to verify isoform B, primers SK124 and SK129 to verify isoform A and primers SK129 and SK130 for verification of mutated isoform A version, were used.

Biochemistry

(Co-) immunoprecipitation. 20ml flies were collected to a falcon tube and frozen in liquid nitrogen. Flies were passed through a metal sieve to obtain heads that were grinded before uptake in 10ml ice cold IP buffer (25mM Hepes KOH, pH8, 150mM NaCl, 1mM EDTA, 0.1% Triton-X100, 1mM DTT, 5mM EGTA, 10mM NaF, 10mM Na pyrophosphate, 20mM B-glycerophosphate, 1mM Na-

vanadate, 1mM PMSF, 1x complete Mini, EDTA free protease inhibitor cocktail, Roche). Lysate was incubated with antibody coupled beads (Affi- Prep Protein A support, BioRad, crosslinked with α Myc 9E10 for IP against Myc- tagged proteins, GFP- Trap, Chromotek for IP against GFP- tagged proteins) for 40min at 4°C, washed at least 6x3 min with ice cold IP buffer and eluted with 40 μ l 200mM Glycine for 30min at RT. Elutes were taken for further analysis (WB, Silverstain, MassSpec)

SDD-AGE was performed as described in (Halfmann and Lindquist, 2008). IP samples were loaded on horizontal 3% TAE Agarose gel containing 0,1% SDS and run for 7hrs at 50V in TAE buffer. Gel was then blotted over night onto nitrocellulose membrane using 1xTBS buffer with 0,1%SDS.

Western Blot. To verify expression of the rescue constructs western blot experiments against the GFP tag were performed. 25 heads per genotype were lysed in homogenization buffer (15mM NaCl, 3mM MgCl₂, 0.1mM CaCl₂, 5% glycerol, 1% Triton- X100, 1x protease inhibitor tablets (Roche, complete Mini, EDTA free protease inhibitor cocktail)) and 15 head equivalents loaded to a Novex NuPAGE 4-12% Bis-Tris-Gel (Invitrogen). The proteins were transferred to a PVDF membrane (Millipore) using semi-dry transfer @12V for 2hrs. Membrane was blocked in 5% milk over night and incubated for 1hr with primary antibody. After 3x10min washes in PBST (PBS+ 0,05%Tween20) membrane was incubated for 1hr in secondary antibody. The membrane was developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Antibodies used: α GFP (Abcam 6556 rabbit polyclonal to GFP, 1: 1000), α Myc (mouse monoclonal 9E10, 1:500), α Orb2 273 (rabbit polyclonal to Orb2, 1:2000, unpublished, gift of Kausik Si), ECL Anti- mouse IgG, Horseradish Peroxidase linked F(ab')₂ fragment (from donkey) (GE healthcare, 1:200 000), ECL Anti- Rabbit IgG, Horseradish Peroxidase linked whole antibody (from donkey) (GE healthcare, 1:200 000).

Silverstain. Silverstain was performed following Blum Silver Staining. Protein elutes were loaded onto a Novex NuPAGE 4-12% Bis- Tris- Gel (Invitrogen). The gel was then fixed for 1hr in 40%EtOH, 10% HAc, washed 2x 20min in 30% EtOH and further 20min in H₂O. The gel was sensitized in 0.02% Na₂S₂O₃ for 1min before incubation in 0.1% AgNO₃ for 20min at 4⁰C. After 3x wash in H₂O the gel was developed in 3% Na₂CO₃, 0.05% formalin. The reaction was stopped with 5% HAc.

Behavioral tests

All flies were raised on semi defined medium at 25°C in a 12 hr:12 hr dark:light cycle. Virgin males were collected at eclosion and aged individually for 5 days before training; females were aged for 3 days together with males in groups of ~100 flies to ensure them to be mated. All assays were performed at circadian time 6:00–10:00, and on at least 3 independent occasions. All alleles were backcrossed to wild type strain w1118 for at least 3 generations before use in behavioral assays.

Courtship Conditioning. Males were placed individually with (training) or without (sham control) a single mated female in a chamber containing food for 7hrs (Siwicki and Ladewski, 2003). After this training period males were separated to a fresh chamber and kept in isolation until test. For testing males were paired with fresh mated females for 10min in a 10mm diameter courtship chamber and videotaped for 10min (JVC handycam, 30GB HD). Videos were scored for the percentage of time each male spent in courting during the 10min test either manually (for Supl. Table 2, 6, 7, 10, 11, 12, 13) or by flytracker generated in the Dickson lab (unpublished Schusterreiter C., Machaczek C., Dickson B.J.) (for Supl. Table 3,4,5,8,9). This Courtship index (CI) was used to calculate the Learning Index (LI): $CI_{naive} - CI_{trained} / CI_{naive} \times 100$. Values are mean $\pm$ SEM. LIs were analysed using a script to calculate permutation test. *P* values are

for H_0 : $LI = LI_{+/+}$ (permutation test) and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for H_0 , $LI = 0$ (permutation test)

Locomotion assay. Homozygous male were placed in empty food vials and allowed to recover from CO_2 for 1hr. After this flies were banged to the bottom of the vial. Percentages of flies leaving the 1st centimeter from the bottom of the vial within half a minute were declared to have no phenotype.

Immunohistochemistry

adult heads, VNC and larval CNS. Fly brain and VNC were separately dissected (between 5 to 8 days after eclosion) in PBS and fixed using 4% paraformaldehyde in PBST (PBS with 0.3% Triton X-100) for 20 minutes at $24^\circ C$. After washing in PBST, the tissues were blocked in 5% normal goat serum in PBST for at least 2 hours. The primary antibody and secondary antibody were incubated for 48 hours at $4^\circ C$. The tissues were washed with PBST 3x 10min and then overnight at $4^\circ C$ between the primary and secondary antibody incubations. After the secondary incubation, samples were washed 3x 10min and overnight at $4^\circ C$ before mounting in Vectashield (VectorLabs).

Antibodies used were: rabbit polyclonal anti-GFP (1: 5000, Torri Pines); mouse nc82 (1:20, Hybridoma Bank); mouse disc large (1:500, Hybridoma Bank); secondary Alexa -488, - 568 antibodies (1: 1000, Invitrogen).

Embryo. Embryos were collected from apple juice plates. Embryos were incubated in 50% bleach for 3min and carefully washed into a sieve using tap water to remove bleach. Embryos were transferred to eppendorf tubes containing 500 μ l heptane. 450 μ l PBS and 50 μ l formaldehyde were added to fix embryos for 20min at RT. Lower phase was removed first, then the heptane and replaced by fresh heptane and ice cold methanol. Then embryos were shaken strongly for 1min to remove their vitelline. Embryos were then washed 3x 5min in methanol followed by 3 washes in PBST to remove residual methanol. Embryos were

blocked for 1hr in 5% normal goat serum prior antibody incubation. Antibody incubation was from 1hr at RT to over night at 4°C. Between primary and secondary antibody incubation, as well as before mounting embryos were washed 4x10min in PBST.

Antibodies used were: rabbit polyclonal anti-GFP (1: 5000, Torri Pines), mouse anti engrailed (1:100, Hybridoma Bank, BP104), mouse anti FasII (1:20, Hybridoma Bank, 1D4), rabbit anti repo (), secondary Alexa -488, - 568 antibodies (1: 1000, Invitrogen).

Confocal microscopy. Tissues were scanned using a Zeiss LSM 510 with a Zeiss Multi Immersion Plan NeoFluar 25X/0.8 objective. On average 8 brains or 5 VNCs were imaged for each genotype. Scanning parameters were set to image the central brain or the entire ventral nerve cord within 30 minutes. Images were taken at 512 X 512 pixels and 180 slices at 1.2µm interval. A macro plug-in was used to automate the scanning process. Images were processed in ImageJ (NIH) to obtain maximum intensity Z projections.

Immuno-EM on *Drosophila* Brains. A protocol for pre-embedding Method has been followed (according to protocols in Yasuyama et al., 2002, J Comp Neurol 445, pp211-226). Briefly 3-4 days old males were dissected and fixed in 4% paraformaldehyde, 0.1% glutaraldehyde, 0.07M phosphate buffer pH 7.3 for 3 hours at 4C. Next heads were cut with a vibratome in slices of 80µm. Slices containing the brain were stained by rabbit anti-GFP (Molecular probes, dilution 1:200) diluted in 5% NGS in PBT and incubate for 44 hours at 4C. Next Vectastain secondary antibody biotinylated goat anti-rabbit IgG at 1:200 dilution in 5% NGS was used to incubate the slices for 20 hours at 4C. Thereafter the brains were incubated with avidin-biotinylated-peroxidase complex for 20hrs at 4C. Then the slices were incubated for 10 min in 200ul DAB solution. Next 200ul H₂O₂ solution (7 ul of 3% H₂O₂ in 1 ml DAB solution) were added and the staining developed. After 3 washes in PBT(triton) a wash in 0.1 M Cacodylate buffer (pH7.4) followed before the slices that are in the right brain area have been

selected. Slices were post fixed in scintillation vial in 0.1% Osmium tetroxide in 0.1M cacodylate buffer pH 7.4 in 0.08% potassium ferrocyanide for 1 hour at 4C and then washed three times with cacodylate buffer. Next the slices were dehydrated in a series of EtOH steps (70%-(transfer to Jos) 80%-90%-100% EtOH). The slices were flat-embed in Epon resin by sandwiching the slices between two Sylon-CT coated object slides using two coverslips as spacers and polymerized together with a number of BEEM capsules filled with Epon. The area of interest was cut out with scalpel from the Epon block (BEEM capsule) and cut into semi-thin slices (0.5-1um thick) and collected on object slide. Slides next were stained with toluidine blue to localize cells/tissue of interest, which was then cut in ultrathin (80 nm) sections. These were collected on EM specimen grids and post-stained with uranyl acetate.

Author contributions

K.K. proposed the project. K.K. and S.K. designed the experiments. B.J.D designed the Orb2^{attP} allele. S.K. and B.J.D designed the general cloning strategy. S.K. generated the orb2 alleles, carried out all studies and did all the analysis. B.S. generated the orb2 S2 cell lines. J.N did the EM studies. K.K. supervised the project.

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3.1 Abbreviations

20E	20-hydroxyecdysone
AC	adenylyl cyclase
AKAP	A kinase anchoring protein
amn	amnesiac
ARM	anesthesia-resistant memory
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CPE	cytoplasmic polyadenylation element
CRE	cAMP response element
CREB	cAMP response-element binding protein
cVA	cis-vaccenyl acetate
DCO	catalytic subunit of cAMP-dependent protein kinase
dnc	dunce
FMRP	Fragile X Mental Retardation Protein
fru	fruitless
IP	immunoprecipitation
LED	light-emitting diode
LEs	localization element
LTM	long-term memory
MB	mushroom body
MTM	mid-term memory
NMDA	N-Methyl-D-aspartate
PACAP	pituitary adenylyl cyclase–activating peptide
PDE	phosphodiesterase
PKA	protein kinase A
PKC	protein kinase C
RBD	RNA binding domain
RNP	ribonucleoprotein

RRM	RNA recognition motif
rsh	radish
rut	rutabaga
UTR	untranslated region
Znf	Zinc finger motif

4. Curriculum Vitae

Personal Information

Name: Krüttner Sebastian

Date of birth: 19.05.1981

Nationality: Austria

E - mail: sebastian.kruettner@imp.ac.at

Education

01/2007- present: PhD student, IMP Vienna, K. Keleman, PhD

09/2005 –12/2006: Diploma thesis (Masters Equivalent)

2003-2006: 2nd section of study of Genetics, University of Vienna

main subject: developmental genetics

elective subjects: gene and biotechnology

bioinformatics

optional subjects: science and media, spectroscopic methods

09/2003: 1st Diplomprüfung (1st Diploma exam)

2001 - 2003 1st section of study of Biology (subject Genetics), University of Vienna

200 - 2001: civil service at the “Arbeiter Samariterbund Salzburg”

1991 - 1999: high school “Bundesgymnasium Salzburg/Nonntal”

1987 - 1991: primary school

Research experience

01/2007- present: PhD student, IMP Vienna, K. Keleman, PhD

“The role of the *Drosophila* CPEB protein Orb2 in long-term memory”

09/2005 –12/2006: Diploma thesis, IMP Vienna, B. Dickson, PhD /K. Keleman, PhD

“ Molecular basis of memory and learning- the role of CPEB in long-term memory”

09/2004 - 08/2005: Research Technician, IMBA Vienna- RNAi laboratory, B. Dickson

“A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*”

06/2004 - 08/2004: “Wahlbeispiel” IMBA Vienna, B. Dickson, PhD and F. Schnorrer, PhD

“Systematic genetic analysis of muscle morphogenesis and function

in *Drosophila*”

Conferences

2011

Poster: “Dissecting the role of *Drosophila* Orb2 in long- term memory”

The EMBO meeting, Vienna

Poster: “Dissecting the role of *Drosophila* Orb2 in long- term memory”

52nd Annual *Drosophila* research Conference, San Diego

2010

Poster: “Dissecting the role of *Drosophila* Orb2 in long- term memory”

EMBO | EMBL Symposium: Structure and Function of Neural Circuits, Heidelberg

2007

Poster: “Function of Orb2 in long-term memory”

20th European *Drosophila* Research Conference, Vienna

2006

Poster: “Function of Orb2 in long-term memory”

5th Forum of European Neuroscience, Vienna

Publications

Dopamine neurons modulate pheromone responses in *Drosophila* courtship learning

Krystyna Keleman, Eleftheria Vrontou, Sebastian Krüttner, Jai Yu, Amina

Kurtovic and Barry J. Dickson, Nature (in review)

Function of the *Drosophila* CPEB protein Orb2 in long-term courtship memory.

Keleman K, Krüttner S, Alenius M, Dickson BJ. Nat Neurosci. 2007 Dec;10(12):1587-93.

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