ERASURE AND OCCLUSION OF A BEHAVIORAL MEMORY BY MUTANT FORMS OF CAMKII SUPPORTS THE ROLE OF CAMKII IN MEMORY MAINTENANCE

Senior Thesis

Presented to

The Faculty of the School of Arts and Sciences
Brandeis University

Undergraduate Program in Biology
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In partial fulfillment of the requirements for the degree of Bachelor of Science

by

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May 2017

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Abstract

The exact mechanism that underlies memory is still one of the biggest unresolved mysteries in neuroscience. However, a strong candidate for this memory mechanism is the activity-based strengthening of synapses known as long-term potentiation (LTP). Calcium/calmodulin-dependent protein kinase II (CaMKII), an important molecule for the induction of LTP, has been shown to play an important role in the maintenance of LTP and, as a result, is thought to be crucial for the process of memory storage. The importance of CaMKII in memory maintenance has not been verified on a behavioral level. Here we show that the transient expression of a dominant-negative form of CaMKII results in the erasure of a behavioral memory. Erasure of the memory persists after the expression of the mutant has ceased; suggesting that a maintenance process mediated by CaMKII was disrupted. To further investigate the role of CaMKII in memory, a constitutively activated form of CaMKII was expressed in the hippocampus in order to occlude memory. Expression of the activated form of CaMKII did, in fact, cause memory deficits thus providing more evidence to the theory that CaMKII plays an essential role in memory.
Introduction

The exact molecular basis of memory has eluded neuroscientists for years and the problem currently remains unresolved. An underlying mechanism for memory has yet to be found and fully verified for all the properties that one would expect a memory mechanism to have. In order for a mechanism to be a viable candidate for the basis of memory it needs to have one important characteristic, it has to be reliably persistent over time. This property is essential for any potential memory mechanism since it would account for the remarkable storage component of memory that is observed; memories need to last over large periods of time. Neuroscientists have discovered a potential explanation to this memory storage problem in the form of long-lasting modifications made to synapses by an activity-dependent process termed long term potentiation (LTP) (Sweatt, 2016). LTP is the strengthening of synapses that occurs after persistent activity, making it the ideal mechanism for the molecular basis of memory.

LTP is typically defined as having three distinct processes: induction, maintenance and expression (Sweatt, 1999). The induction phase of LTP begins with the release of glutamate from the presynaptic cell. This neurotransmitter release is coupled with the depolarization of the postsynaptic cell in order to relieve the Mg$^{2+}$ block of the NMDA channels that is present at hyperpolarized potentials. Once the NMDA channels are activated and opened, calcium ions flow into the cell and intracellular calcium levels increase. This spike in calcium concentration causes the activation of various proteins, such as CaMKII and PKMζ (Luscher and Malenka, 2012). Activation of these proteins leads to a cascade of events that result in the recruitment of AMPA channels to the synapse, thus strengthening the synapse.
The structure and role of CaMKII (Ca\(^{2+}\)/calmodulin-dependent protein kinase II) in LTP and AMPA channel recruitment has been extensively studied. The overall protein structure of CaMKII is that of a dodecamer, it is composed of 12 CaMKII subunits organized into two 6-subunit rings stacked on top of each other (Kolodziej et al., 2000). Each of these subunits has three distinct domains: the kinase domain (phosphorylates substrates), the regulatory domain (regulates kinase activity), and the hub domain (links CaMKII subunits together). These domains all play vital roles in the proper function of CaMKII (Stratton et al., 2013). CaMKII is activated when calmodulin, a calcium-binding messenger, binds to the regulatory domain (specifically at the calmodulin (CaM) footprint) of a subunit as a response to calcium influx into the cell (Payne et al., 1988; Stratton et al., 2014). When CaM is not bond to the regulatory domain, the regulatory domain covers the kinase domain thus preventing catalytic activity. Once CaM is bond the regulatory domain will dislodge from the kinase domain of the subunit (Colbran et al., 1989; Yang and Schulman, 1999) allowing CaMKII to phosphorylate its substrates. The activation of the kinase results in the aforementioned signaling cascade that ends with recruitment of AMPA channels to the synapse, thus resulting in overall strengthening of the synapse.

CaMKII, as well as PKMζ, are essential molecules in the induction of LTP (Lisman et al., 2002; Lisman et al., 2012; Sacktor, 2008, 2012). Experimental evidence initially supported the idea that these proteins were important for just the induction of LTP (Malinow et al., 1989) but it has been recently postulated that these proteins could play a role in LTP maintenance as well (Pastalkova et al., 2006; Sanhuez et al., 2011). In order for LTP to be a valid candidate for the underlying mechanism of memory, its maintenance process needs to be persistent and resilient in order to account for the exceptional time frame of memory that is observed in nature. As a result,
proteins that play in a role in LTP maintenance are expected to have specific biochemical properties that would allow LTP to remain active over the long periods of time that are needed for a potential memory mechanism (Lisman, 1985). CaMKII possess three unique biochemical properties that make it a strong candidate to be the pivotal protein responsible for the maintenance of LTP or, in other words, a potential memory molecule (Lisman and Goldring, 1988a; Lisman and Goldring, 1988b). In order for CaMKII to remain persistently activated over long periods of time, CaM would have to remain constantly bound to the regulatory domain. Biochemically this could be considered impossible since CaMKII has one of the lowest affinities (Kd ≈ 15 nM) for CaM out of all CaM-binding proteins (Meyer et al., 1992). To overcome this biochemical barrier and to prevent CaM from falling off, and the subsequent inactivation of the protein, CaMKII has developed a feature that allows it not only increase its affinity for CaM (Kd= 20 pM) but also mimic the properties of CaM binding (Meyer et al., 1992). In its regulatory domain, CaMKII has phosphorylatable amino acid residue, T286. The T286 site is phosphorylated upon binding of CaM to the regulatory domain (Hanson et al., 1994). The phosphorylation of T286 allows CaM to fall off from regulatory segment while also preventing the rebinding of the segment to the kinase domain (Buard et al., 2010; Coultrap et al., 2010; Giese et al., 1998). With CaM binding no longer needed to activate the kinase, CaMKII is considered autonomous (i.e. independent of calcium activity).

Two other amino acid residues in the regulatory domain, T305/T306, also play a role in this autonomous process. These two residues are in the CaM footprint region of the regulatory domain and, upon phosphorylation, directly interfere with the binding of CaM to the region (Patton et al., 1990). Once CaM dissociates from the CaM footprint region, T305/T306 are phosphorylated to prevent any further CaM binding (Hanson and Schulman, 1992). Upon
phosphorylation of all three of these amino acid residues, CaMKII enters an autonomous state where it can remain active regardless of neural activity (Saitoh and Schwartz, 1985).

Since the autonomous property of CaMKII is reliant on phosphorylation, it stands to reason that endogenous levels of phosphatase could result in the deactivation of CaMKII. However, CaMKII has another biochemical property that prevents this from occurring, autophosphorylation (Miller and Kennedy, 1986). Upon CaM binding, T286 phosphorylated subunits can perform intraholoenzyme phosphorylation reactions on neighboring subunits at the T286 residue (Lai et al., 1986). This allows the kinase to remain active if one subunit is dephosphorylated or if one subunit is replaced by a non-phosphorylated subunit.

Subunit exchange is another critical feature of CaMKII function. It can be used to place novel subunits into an already active CaMKII holoenzyme, as mentioned above, but it can also be used to propagate CaMKII activation. T286 phosphorylated subunits can leave the CaMKII holoenzyme and place themselves into an inactivated CaMKII holoenzyme (Stratton et al., 2014). These subunits then go on to phosphorylate their neighboring subunits and the holoenzyme becomes active, without any CaM activity. Together the autophosphorylation, autonomous, and subunit exchange properties of CaMKII provide it with the essential biochemical features that you would expect in a memory molecule (Lisman and Goldring, 1988a; Lisman and Goldring, 1988b).

There is experimental evidence to support the idea that PKMζ and CaMKII both play important roles in the process of LTP maintenance. It has been shown that the application of a PKMζ inhibitor, whose selectivity for the protein has been questioned (Volk et al., 2013), to hippocampal slices 3 hours after induction of LTP could result in the reversal of LTP (Serrano et al., 2005). This experiment has been reproduced on a behavioral level where application of the
PKMζ inhibitor resulted in the erasure of a CA1-dependent behavioral memory (Pastalkova et al., 2006). Similar experiments have been conducted to show that CaMKII also plays a role in LTP maintenance. The application of a CaMKII inhibitor peptide that mimics portions of the regulatory segment (Sanhueza et al., 2011) resulted in the reversal of LTP. Importantly, it was also shown that LTP could be reinduced after the inhibitor was washed out. This indicates that a maintenance process was being disrupted, rather than an expression process. As has been shown, if a protein is involved in the maintenance process of LTP then disrupting its function should result in reversal of LTP.

The erasure of LTP by CaMKII interference has been shown in slices but no experiments have been conducted on the behavioral level. Thus to further investigate the role of CaMKII in memory maintenance, it is important to conduct these erasure experiments on behavioral level. A strict criteria needs to be followed when investigating the role of a protein in a maintenance process. First, the inhibitor that is being used to disrupt the function of a specific protein must be applied after the process (LTP) is induced; this ensures that any erasure that occurs is not due to disrupting induction. Second, the inhibitor must be taken away before expression of the process is checked. In the case of memory maintenance this means that the inhibitor needs to be removed before retention of the memory is tested. If erasure of the memory occurs in the absence of any inhibitor then it can be assumed that the inhibitor was disrupting a maintenance process and not an expression process. Lastly, re-induction (learning of the behavioral memory) of the process definitively shows that a maintenance process was disrupted, since any interference of induction or expression processes would result in relearning deficits. In order to fulfill the above criteria, it was decided that a Herpes Simplex Virus (HSV) vector would be chosen to express the inhibitor for our erasure experiments. HSV exhibits transient expression properties (Carlezon et al., 1997;
Neve et al., 2005), which would allow retention of the behavioral memory to be tested after the inhibitor was no longer being expressed.

It is believed that memories are formed in neural networks by patterns of synaptic strengthening (Hopfield, 1984). The goal of the erasure experiments is to destroy this pattern by reversing the changes in synaptic strength that occurred as a result of learning a behavioral task. In our experiment, the dominant-negative K42M mutation of CaMKII (Kuhl et al., 2000; Yamagata et al., 2009) was the chosen inhibitor that would be used to cause the reversal of synaptic strength. The K42M mutation is a mutation in the ATP-binding pocket of the kinase domain (O’Leary et al., 2011), this means that ATP can not bind to the kinase, preventing the kinase from phosphorylating its substrates. We theorized that upon expression, the mutant K42M-CaMKII subunits would integrate into existing activated CaMKII holoenzymes, based on the subunit exchange properties of CaMKII (Stratton et al., 2014). Their inability to phosphorylate would result in the breakdown of the autophosphorylation feature of CaMKII, resulting in a deactivation of the holoenzyme. In theory, this would reverse the synaptic strengthening caused by the activation of CaMKII upon learning. The K42M mutation has been used previously to show that CaMKII function is necessary in memory; knock-in expression of the dominant-negative mutation was successful in reducing a hippocampal-dependent memory (Yamagata et al., 2009). This mutation has also been expressed in the dentate gyrus (DG) to reverse a form of addiction (Loweth et al., 2013), indicating that it has the potential to erase a behavioral memory as well.

For the behavior experiments, the conditioned place-avoidance task was selected as the behavioral memory. This task has been shown to be CA1-dependent as disruptions in the CA1 region have resulted in memory deficits (Pastalkova et al., 2006). After the animals learned the
behavior task, injections of HSV-K42M (HSV vector expressing the K42M mutation of CaMKII) were made into the CA1 region of the hippocampus. If successful, the transient expression of K42M-CaMKII (Carlezon et al., 1997; Neve et al., 2005) would result in the erasure of the behavioral memory, essentially making it appear like the conditioned place-avoidance task was never originally learned. If erasure of this memory is achieved than it would provide concrete behavioral evidence that CaMKII is essential in the process of memory maintenance.

Similar to the Sanhueza et al. 2011 and Serrano et al. 2005 experiments mentioned above, there is an abundant amount of electrophysiology experiments that have been conducted that show if synaptic strength is saturated then subsequent LTP induction is significantly inhibited (Lledo et al., 1995; Pettit et al., 1994; Pi et al., 2010). However, as with the erasure experiments, these occlusion experiments with CaMKII have not been conducted on a behavioral level.

As discussed, memory is thought to be encoded by the particular subset of synapses that are strengthened (Hopfield, 1984). In line with this theory, occlusion experiments show that widespread induction of LTP in the dentate gyrus, which would destroy this pattern, can degrade a memory (Brun et al., 2001). On a behavioral level, injecting an HSV vector expressing an activated form of CaMKII after an animal has learned the conditioned place-avoidance task would be expected to mimic these results. In our occlusion experiments, the triple mutant T286D/T305A/T306A was chosen as the activated form of CaMKII. The aspartate (D) mutation mimics phosphorylation at the T286 site, this allows the CaMKII to become autonomous and independent of CaM activity. The alanine (A) mutations at T305/T306 prevent these sites from being phosphorylated (Pi et al., 2010). Since phosphorylation at these sites result in CaM being unable to bind to the regulatory domain, preventing these sites from being phosphorylated would
allow CaM to stay bound and/or freely bind to the CaMKII subunit, thus producing constant activity. Experiments in hippocampal slices showed that this activated form of CaMKII saturates LTP (Pi et al., 2010). Phosphorylation of T286 has been previously shown to promote translocation of CaMKII to the PSD (Rich and Schulman, 1998; Strack and Colbran, 1998) and to promote subunit exchange (Stratton et al., 2014) thus allowing this activated form to propagate through the cell and activate other inactivated CaMKII holoenzymes, regardless of Ca\(^{2+}\) activity.

If CaMKII does, in fact, play an important role in memory then expressing a constitutively activated form of the kinase would result in synaptic saturation, all the neurons within the region would be potentiated and any pattern of potentiation would be destroyed. Thus if this experiment was successful then the results would be similar to the erasure experiments, the retention test would show that the animal forget the behavioral task.
**Materials and Methods**

**Conditioned Place-Avoidance Training:** Adult male Long-Evans rats between 2- and 3-months-old were used for conditioned place-avoidance training. The animals were housed 1-2 per cage before training (1 per cage after training) in a temperature-controlled room with a 12-12hr light/dark cycle. The animals were placed on a circular platform (82 cm in diameter) that rotates counterclockwise at 1 rpm. The position of the animal was monitored using software (Tracker 2.33 Bio-Signal Group Co.) that analyzes images from an overhead camera and delivers a shock when the animal was detected in a shock zone defined relative to the visual cues placed on the wall (Fig. 1A). A mild shock (500 ms, 60 Hz, 0.3 mA) was delivered via the electrified grid on the platform floor to stimulate avoidance but not a freeze response by the animal. If the animal was not responding to the 0.3 mA shocks, the current was increased to 0.4 mA. If the animal did not respond to 0.4 mA, the experiment was discontinued and the animal was sacrificed. Shocks were not increased about 0.4 mA for fear of inducing freezing of the animal. Animals that entered the shock zone were shocked repeatedly at 1.5-second intervals until they left the shock zone.

On Day 1, a 10-minute pretrial was given without shock to habituate the rat to the apparatus (data omitted from results) and then six 10-minute trials (Trial 1-6) with shock were performed (with 10-minute break in the home cage between each trial). On Day 4, six 10-minute trials (Trial 7-12) with shocks were repeated. If an animal did not show apparent learning after the first and/or second day, the experiment was discontinued and the animal was sacrificed. On Day 7, virus was injected. For erasure experiments, the retention tests of the behavioral task were conducted on Day 17 in a single 10-minute trial (Trial 13) with shock. For the Occlusion experiments, the retention tests were conducted on Day 10 with a single 10-minute trial (Trial
13) with shock. After trial 13, relearning was measured in three 10-minute trials (Trials 14-16) for both the erasure and occlusion tests. All behavioral experiments were done blind, the person conducting the experiment was not aware of the virus injected into the animal.

**Injection:** Herpes Simplex Virus (HSV) injection was performed on Day 7 for both erasure and occlusion experiments. Animals (300-500 g at time of surgery) were anesthetized with a combination of ketamine (100 mg/kg, ip) and xylazine (2.5 mg/kg, ip) and were placed in a stereotoxic apparatus (Kopf Instruments). Bilateral injections of the virus (AP: -3.5, ML: ±2.0, DV: -2.7 (and -3.0) and AP: -5.3 ML: ±2.6 DV: -2.2 (and -2.5)) were done, with the primary target being the CA1 region of the hippocampus. A total of eight injections were made at four injection sites, with two injections of different depths at each site, often leading to the deepest injection producing expression in the dentate gyrus (Fig. 2A). Each injection (Nanoject II, Drummond Sci.) consisted of fourteen 50 nL volumes of virus with 6 seconds between each volume. Overall for each injection, 0.7 uL of the virus was injected over the course of 1.5 minutes. After each injection, the glass pipette was left in place for 5 minutes in order to facilitate diffusion of the virus. All surgery was conducted with aseptic technique in accordance with Brandeis University and IACUC approved protocols.

**HSV vector:** HSV viral vectors with inserted genes (GFP, mutant CaMKII) were obtained from Rachael Neve at the MIT Viral Core Facility. Expression of the T286D/T305A/T306A mutant CaMKII gene (fused with GFP) was controlled by the HSV IE 4/5 promoter. Expression of the K42M mutant CaMKII gene was similarly controlled by the HSV IE 4/5 promoter, with GFP expression being separately controlled by the CMV promoter. Both virus vectors resulted in
co-expression of GFP and the desired mutant CaMKII. For the virus only expressing GFP, no
target gene was inserted into the vector and the CMV promoter drove expression of GFP.

**Immunohistochemistry:** To measure viral expression, three days after virus injection (occlusion experiments) and 10 days after virus injection (erasure experiments), intracardial perfusion was performed and brains were fixed in a 4% paraformaldehyde solution. After a day in 4% formaldehyde, brains were placed in a 0.1M PBS solution before being sectioned a day later. Hippocampal slices of the CA1 region were obtained using a vibrating blade microtome, Leica VT1000 S (Leica Microsystems Inc). Expression of HSV proteins was verified by immunostaining of GFP. 1:5000 chicken anti-GFP [Aves Laboratories] primary antibodies and 1:500 goat anti-chicken alexa^488 [Invitrogen] secondary antibodies were used to enhance fluorescence of GFP. By comparing GFP fluorescence with NeuN staining, 35-50% of neurons were found to be transfected around the injection site (~1 mm). For the NeuN staining, 1:1000 mouse anti-NeuN [EMD Millipore] were used as the primary antibodies with 1:500 goat anti-mouse alexa^594 [Invitrogen] as the secondary antibodies. For both staining procedures slices were mounted with DAPI mounting medium [Vectashield], in order to stain the cell bodies. For erasure experiments, to verify the injection sites after the retention test was conducted (GFP is no longer expressed being expressed (Carlezon et al., 1997; Neve et al., 2005), sterile 0.5 µm red fluorescent beads (Mol.Probes) were co-injected. Beads were excited at 580 nm. In a small fraction of experiments, beads could not be detected in sections and these experiments were eliminated from consideration. Fluorescent images were captured using a Keyence Fluorescence Microscope and were analyzed with the Fiji ImageJ image-processing software.
Statistics: A two-sample Kolmogorov–Smirnov test was used to determine whether distributions were statistically different for behavioral data. Analysis was done in MATLAB. Difference was determined to be significant if p-value < 0.5.
Results

Conditioned Place-Avoidance Training Paradigm Results in Long-Term Retention of Behavioral Memory

In our experiments a conditioned place-avoidance task, a task that is hippocampal CA1-dependent (Pastalkova et al., 2006), was chosen as the behavioral task. In this task, an animal is placed on a counterclockwise-rotating circular platform that leads the animal into the designated shock zone, a triangle shaped region (Fig. 1A) that is defined by different colored cues placed on the wall behind the apparatus. In order to study CaMKII’s role in memory maintenance on a behavioral level, the animal needs to show retention of the behavioral memory for an extended period of time. If the training paradigm that was designed did not result in long-term retention of the memory then the animal simply forgetting the task could be interpreted as erasure of the memory. A specific training paradigm was designed in order to achieve long-term retention of the behavioral task in the animal (Fig. 1B). On the first day of training animals were trained for six 10-minute trials (Day 1, Trials 1-6) with another set of six 10-minute trials taking place three-days later (Day 4, Trials 7-12). The first day of training also consisted of a 10-minute pre-trial in which the animal was allowed to explore the circular platform without being shocked (data excluded from figures for clarity). At the end of Day 4 the animals learned to avoid the shock zone; the average number of shocks per trial fell from approximately 18 in trial 1 to approximately 1 in trial 12 (Fig. 1C). A small increase in number of shocks is observed during trial 7, the first trial of the second day of training. This brief increase in number of shocks is mostly likely due to the animal still learning the behavioral task at this point in the training, it can be assumed that the first day of training was not sufficient to produce a strong retention of the memory. As shown in Fig. 1B, all animals received two days of training (Day 1 and Day 4)
Fig. 1. Experimental Design and Verification of Conditioned Place-Avoidance Behavioral Task: A. Apparatus for conditioned place avoidance, surrounded on all four sides (4th wall side not shown) by visual cues. Circular platform rotates counterclockwise; rotation brings animals into shock zone (outlined by a red triangle). Shock zone is fixed in relation to visual cues. B. Training paradigm for conditioned place-avoidance task. On the first day of training (Day 1) animals received six 10-minute trials (Trials 1-6) with a subsequent six trials on the second day of training (Day 4, Trials 7-12). Animals then received viral injection of HSV vector on Day 7, three-days after the last training session. Retention test was conducted on Day 17 or Day 10, depending on experimental group of animal (Erasure or Occlusion). C. Animal learned the conditioned place-avoidance task over the two training days, as evident by the decrease in number of shocks that is observed. Since training should be similar for all animals used in the experiment, the data represents a summation of all animals used over the course of the occlusion and erasure experiments (n=18). D. Memory of behavioral task was tested on Day 10 (n=2) and Day 17 (n=6) for animals injected with the control virus (empty virus vector with GFP attached). Number of shocks is low for both day 10 and day 17 thus showing retention of behavioral memory is similar for the two different days. There was concern that conducting the retention test three days post-surgery (Day 10) would impair memory, these results show there are no residual effects of surgery on day 10. Two-sample K-S test was used to determine statistical significance between day 10 and day 17 trials. Error bars represent mean ± SEM.
which were followed by injections of the viral vector three-days later (Day 7). After the
injections were made, depending on the type of experiment, retention of the behavioral memory
(retention test) was checked on either Day 10 (Occlusion) or Day 17 (Erasure). Control animals
were injected with HSV-GFP and, regardless of when the retention test was conducted, showed
excellent memory retention with the number of shocks being low in both cases (Fig. 1D). This
indicates that any memory deficits that occur in the experimental animals is a result of mutant
CaMKII expression and not a result of a natural loss of memory or, in the case of Day 10
retention tests, a result of any immediate detrimental aftereffects of the viral injection surgical
procedure. The low number of shocks seen during trial 13 on Day 17 and Day 10 for control
animals (Fig. 1D) also indicates that any long-term aftereffects of viral injection (i.e. damage to
the hippocampus) cannot be the cause of any erasure/occlusion of memory.

**Herpes Simplex Virus Vectors Exhibit Transient Protein Expression Properties**

On Day 7, three days after Day 4 training, a Herpes Simplex Virus (HSV) viral vector
expressing a mutant form of CaMKII (K42M for erasure experiments or T286D/T305A/T306A
for occlusion experiments) was injected into the CA1 hippocampal region of the animals. The
CA1 region was chosen as the injection site since it has been shown that the conditioned place-
avoidance behavioral task was a CA1-dependent memory (Pastalkova et al., 2006). Due to the
properties of the HSV vector (See Methods), green fluorescent protein (GFP) was co-expressed
along with the intended CaMKII mutants. The expression of GFP provided a means to confirm
viral expression and view the extent/area of viral transfection via immunostaining. As seen in
Fig. 2A, strong GFP expression (green in Fig. 2A) can be seen in the CA1 region and DG of the
hippocampus when immunostaining of hippocampal slices was done on Day 10. However, when
Fig. 2 Properties of Viral Expression: A. HSV-mediated GFP expression (green) and co-injected fluorescent beads (red). Expression was high in CA1 and the dentate at three days post-viral injection (top row) but not ten days after (bottom row), in accordance with known transient expression properties of the herpes simplex virus (Carlezon et al., 1997; Neve et al., 2005). Scale bar is 500 microns. Images were taken at 4x magnification.
immunostaining was completed on Day 17 (ten days post-viral injection) no GFP expression was seen in the hippocampus. This observation is in accordance with known transient expression properties of the herpes simplex virus (Carlezon et al., 1997; Neve et al., 2005) with viral expression usually peaking around eight days post transfection. Due to the transient nature of the virus, it is difficult to confirm the presence of the virus or the injection site in erasure experiments since immunostaining occurs on Day 17 (after the retention test is conducted). To solve this problem, red fluorescent beads (red in Fig. 2A) were used to view the sites of injection for immunostaining done on Day 17. This allowed verification that the virus was injected correctly into the CA1 region.

**Expression of the Dominant-Negative K42M-CaMKII Mutant Resulted in Erasure of a Behavioral Memory**

The HSV vector was chosen over the commonly used AAV vector (McCown, 2011) specifically for its transient properties; these properties were needed to successfully conduct the erasure experiments. In order to conclude that an inhibitor is altering memory maintenance, the inhibitor needs to be applied for a period of time after the memory is formed and when the retention of the memory is tested the inhibitor needs to no longer be present. This ensures that if erasure of the memory is observed then it is due to a disruption in a maintenance process of memory, and not the induction or expression (Sanhueza et al., 2011). If AAV was used instead of HSV, then the expression of K42M-CaMKII would never cease (McCown, 2011) and the exact mechanism of how the K42M mutation erased the behavioral memory would be unknown. Due to the use of HSV as the virus vector, K42M was only expressed for approximately eights after viral injection on Day 7. For erasure experiments the retention tests were conducted on Day 17,
**Fig. 3. Erasure Test.** **A.** Representation of the movement of animals during the training trials. Grey lines indicate the path of the animal on the circular platform throughout the ten minutes of the trial. Shock zone is represented by the red triangle with the red dots within the shock zone indicating where an animal received a shock. Figure shows the superposition of the paths of six animals during the first trial of Day 1, the last trial of Day 4 and the first trial of Day 17 (retention test). Top row depicts data taken from control (HSV-GFP) animals; avoidance of shock zone on Day 17 indicates memory retention. Bottom row shows data compiled from erasure experimental animals (HSV-K42M). The high number of shocks seen in the Day 17 superposition indicates that the memory was erased in these animals. **B.** The data seen in Fig. 3A was quantified and summarized for clarity. Two-sample K-S test was used to determine statistical significance (p=0.012). Error bars represent mean ± SEM. N=6 for both control and experimental animals.
meaning the viral proteins were no longer being expressed (Fig. 2A). This allowed for an accurate assessment of whether or not expression of K42M-CaMKII was disrupting a maintenance process.

For the erasure experiments, the well-characterized domain-negative K42M-CaMKII mutant (Kuhl et al., 2000; Xiao et al., 2005; Yamagata et al., 2009) selected for use as the inhibitor. The K42M mutation has been shown to disrupt a wide variety of CaMKII activities such as translocation to the PSD (O'Leary et al., 2011), basal phosphorylation levels (Yamagata et al., 2009), and LTP induction (Yamagata et al., 2009). There is a precedent of the K42M mutation erasing a behavioral memory; it was shown that expression of K42M in the DG could result in the reversal of addiction (Loweth et al., 2013). The proposed mechanism of action for K42M-CaMKII is as follows: erasure is a result of K42M CaMKII subunits that exchange with activated (phosphorylated) CaMKII holoenzymes that were activated upon learning the behavioral task. These subunits lack the ability to bind ATP (catalytically-dead) and cannot phosphorylate any substrates of the kinase; this includes any intraphosphorylation of the T286 residue. Integration of the K42M CaMKII subunits would effectively inhibit the autophosphorylation capabilities of the CaMKII holoenzymes. Without autophosphorylation, the previously activated CaMKII holoenzymes will be deactivated resulting in a reverse of synaptic strength. This reversal would disrupt any patterns of potentiation that may form the behavioral thus causing erasure of the behavioral memory.

It was found that transient expression of K42M-CaMKII in the CA1 region effectively erased the behavioral memory (Fig. 3A). The successful erasure of the behavioral memory is verified by the high number of shocks observed in the first trial of the retention test (p=0.012,Fig. 3B), this indicates that the animals forgot the learned task. After the first trial of
the retention test was conducted, three additional training trials were done in order to access the relearning capabilities of the animals. If the erasure of the memory were a result of damage to the hippocampus that occurred during the viral injection surgical procedure then it would stand to reason that the animals would show deficits in relearning as well as retention of the behavioral memory. Animals were able to relearn the conditioned place-avoidance task and the number of shocks returned to around the same values seen before the viral injection took place (Fig. 3B). Control animals, animals injected with HSV-GFP, showed a low number of shocks in the first trial of the retention test, indicating that they successfully retained the memory of the behavioral task.

**Expression of the Activated CaMKII Mutant Resulted in Occlusion of a Behavioral Memory**

It is thought that memories are formed in the brain when specific neurons are activated in a certain pattern that is particular to that memory (Hopfield, 1984). We previously showed that erasing this pattern results in the loss of a behavioral memory (Fig. 3). Erasing the pattern is not the only effective way to degrade a memory, it stands to reason that if all the synapses around the pattern are activated then the pattern would effectively be destroyed and the memory would be forgotten. It has been shown that inducing LTP in the dente gyrus could disrupt a behavioral memory via synaptic saturation (Brun et al., 2001). Our occlusion experiments tested the hypothesis that an activated form of CaMKII could be used as a means to saturate synaptic activity and occlude a behavioral memory. The triple mutant T286D/T305A/T306A was chosen as the activated form of CaMKII since it has been previously shown to induce LTP in hippocampal slices and saturate the synapses (Pi et al., 2010).
Fig. 4. Occlusion test. A. Superposition of the path of six animals. Grey lines are the paths of the animal, red triangle is shock zone, and red dots are shocks given to the animals. Trial 1 is first training trial on day 1; trial 12 is last training trial on Day 4, and Trial 13 is the first trial on Day 10 (retention test). Control animals (top row, HSV-GFP) retain memory when tested but Occlusion experimental animals (bottom row, HSV-T286D/T305A/T306A) show poor memory retention, as evident by the high number of shocks (red dots) that is observed. B. Data presented in Fig. 4A was quantified and summarized for clarity. Day 10 data (N=8) is a combination of data obtained from retention tests conducted on Day 17 (N=6, see Fig. 3B) and Day 10 (N=2). Day 10 and Day 17 retention data are not significantly different from each other (Fig. 1D). A two-sample K-S test was used to determine statistical significance (p=0.02). Error bars represent mean ± SEM.
For the occlusion experiments, the time of retention was changed from Day 17 to Day 10 since viral expression only lasts for eight days, with protein expression peaking around the fourth day post-injection. Unlike the erasure experiments, the activated CaMKII needed to be present during the retention test since any saturation that was a result of the activated form of CaMKII would possibly dissipate by Day 17, when the virus was no longer expressing. Despite Day 10 retention tests taking place three-days after the viral injection, the results show that the number of shocks seen in control animals when the retention test is conducted on Day 10 is similar to the number of shocks seen in control animals when the retention test is conducted on Day 17; no significant difference was found between Trials 13-16 of Day 10 and Day 17 retention tests (Fig. 1D). This strongly indicates that any increase in the number of shocks seen during the first trial of the retention test for experimental animals would not be due to any aftereffects of the viral injection surgical procedure.

Animals that were injected with the HSV-T286D/T305A/T306A virus showed strong deficits in memory when the retention test was conducted on Day 10 (Fig 4, p=0.02), compared to the number of shocks seen during trial 13 for HSV-GFP injected animals. The strong impairment of memory provides evidence to the theory that saturating all the synapses would disrupt the pattern that is responsible for the formation of a memory. The results from this experiment also strongly support the idea that CaMKII is involved with the normal process of memory storage and is important in strengthen certain synapses to form the pattern needed for memory formation.
Discussion

Since the discovery of long-term potentiation (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973) it has been considered the leading candidate for the molecular basis of memory, specifically hippocampal-dependent memory (Luscher and Malenka, 2012). Over the years, extensive research has been conducted in order to determine the molecular mechanisms that control this activity-dependent strengthening of synapses occurs (Sweatt, 1999). In brief summary, it was found that synaptic strengthening was the result of AMPA channel recruitment to the synapse, which is initiated by the influx of calcium through NMDA channels (Coultrap and Bayer, 2012). CaMKII and PKMζ are two protein kinases that have been found to play important roles in the process AMPA channel recruitment; specifically these two proteins are crucial to the process of LTP induction. Indeed, interference of either protein has been found to lead to learning and memory deficits (Lisman et al., 2002; Lisman et al., 2012; Sacktor, 2008, 2012). These findings have lead to the formation of multiple hypotheses for how these proteins, and others, could play a role in the molecular mechanisms that underlie memory, and as a result the search for definitive proof of a memory molecule commenced (Frankland and Josselyn, 2016; Kandel, 2012; Sanhueza and Lisman, 2013).

PKMζ seemed to be proven as the definitive memory molecule when a series of electrophysiological and behavioral experiments showed that when a PKMζ inhibitor was applied after the induction of LTP and learning, then LTP can be reversed and a behavioral memory could be erased (Pastalkova et al., 2006; Serrano et al., 2005). These experiments seemed to prove that interfering with PKMζ function resulted in the disruption of a memory maintenance process, leading to the assumption that a PKMζ-dependent process was a key
feature underlying memory and learning. The results of these experiments have been recently contested, with the selectivity of the PKMζ inhibitor being put into question (Volk et al., 2013).

Similar electrophysiology experiments have been conducted to determine if CaMKII plays a role in the process of memory maintenance, it was found that applying a CaMKII inhibitor after the induction of LTP resulted in the reversal (erasure) of LTP, subsequent reinduction of LTP was possible after the washing out of the inhibitor (Sanhueza et al., 2011). This indicates that the CaMKII inhibitor was indeed disrupting a LTP maintenance process. This erasure experiment has not been reproduced on a behavioral level, in other words it has not been experimentally confirmed if interfering with CaMKII function can disrupt the maintenance of a memory-dependent behavior. Our experiment is the first to show the erasure of a behavioral memory, as a result of CaMKII interference (Fig. 3). Our results show that transient expression of K42M-CaMKII (a kinase-dead dominant-negative mutation) can result in the erasure of a behavioral memory (Fig. 3), thus indicating that CaMKII is responsible for the maintenance of a behavior-dependent memory. Successful erasure of the memory adds support to the CaMKII hypothesis for memory maintenance, since erasure of the memory makes it highly likely that a CaMKII-dependent process maintains the memory of the conditioned place-avoidance task.

The CaMKII hypothesis for memory maintenance is also strongly supported by the unique biochemical and structural properties of CaMKII: autonomy (Coultrap et al., 2010), autophosphorylation (Giese et al., 1998), and subunit exchange (Stratton et al., 2014). As outlined above, CaMKII holoenzymes phosphorylated at the T286 residue can undergo subunit exchange with unphosphorylated (inactive) holoenzymes (Stratton et al. 2014). These activated subunits can then phosphorylate neighboring subunits (autophosphorylation) in the inactivated holoenzymes, thus leading to activation of the CaMKII (Giese et al., 1998). This would allow
CaMKII to remain activated despite protein turnover and endogenous phosphatase activity. This proposed mechanism could explain how CaMKII can remain activated for long periods of time after learning/memory formation. The results from our erasure experiments verify the idea that an activated form of CaMKII is needed for memory maintenance, since incorporation of a kinase-dead (K42M) subunit would result in the subsequent deactivation of the CaMKII holoenzymes. This would be due to the inability of the kinase-dead subunits to undergo autophosphorylation, leaving the CaMKII holoenzymes that were activated upon learning of the behavioral task susceptible to inactivation by endogenous phosphatase and protein turnover.

The importance of the activated form of the CaMKII holoenzymes in memory is further highlighted by the results of our occlusion experiments. Transient expression of the activated form of CaMKII (T286D/T305A/T305A mutation) resulted in the occlusion of a behavior-dependent memory (Fig. 4). If the proposed mechanism is correct and activated CaMKII holoenzymes do play a significant role in synaptic strengthening and memory (i.e. forming patterns of potentiation) then expression of an activated form of CaMKII would result in synaptic saturation, effectively destroying any pattern that may have formed during learning. These results align with previous studies that have shown that interfering with CaMKII can result in memory deficits ((Hinds et al., 1998; Malinow et al., 1989; Silva et al., 1992)). Overall, the results from the occlusion experiments validate the activated CaMKII mechanism put forward for memory maintenance. On a larger scale, the results from the occlusion experiments also provide more evidence for CaMKII’s role in memory.

Showing the erasure and occlusion of a memory-dependent behavior has strengthened the hypothesis that a CaMKII-dependent process is responsible for the maintenance of memory, as well as providing more evidence for CaMKII’s role as the memory molecule. However, the
answer to the question of “What is the molecular mechanism of memory?” may not be this simple. It is likely that the complex process that is memory is not simply controlled by only one molecule but rather is the result of a multitude of large-scale and synapse-specific processes working together to maintain and form memories within the brain (Kandel et al., 2014). However, the revelation that CaMKII can serve as a molecular memory, even just for CA1-dependent memories, provides great insight into the normal (or abnormal) mechanisms that underlie memory. Understanding CaMKII’s role in memory will open up other avenues of exploration and may even lead to a deeper understanding of the mysterious and complex process called memory.
References


