HHS Public Access

Author manuscript

*Neurobiol Learn Mem*. Author manuscript; available in PMC 2018 October 10.

Published in final edited form as:

*Neurobiol Learn Mem*. 2017 January ; 137: 134–141. doi:10.1016/j.nlm.2016.11.018.

# Differential role of calpain-dependent protein cleavage in intermediate and long-term operant memory in *Aplysia*

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## Abstract

In addition to protein synthesis, protein degradation or protein cleavage may be necessary for intermediate (ITM) and long-term memory (LTM) to remove molecular constraints, facilitate persistent kinase activity and modulate synaptic plasticity. Calpains, a family of conserved calcium dependent cysteine proteases, modulate synaptic function through protein cleavage. We used the marine mollusk *Aplysia californica* to investigate the *in vivo* role of calpains during intermediate and long-term operant memory formation using the learning that food is inedible (LFI) paradigm. A single LFI training session, in which the animal associates a specific netted seaweed with the failure to swallow, generates short (30 min), intermediate (4–6 hr) and long-term (24 hr) memory. Using the calpain inhibitors calpeptin and MDL-28170, we found that ITM requires calpain activity for induction and consolidation similar to the previously reported requirements for persistent protein kinase C activity in intermediate-term LFI memory. The induction of LTM also required calpain activity. In contrast to ITM, calpain activity was not necessary for the molecular consolidation of LTM. Surprisingly, six hours after LFI training we found that calpain activity was necessary for LTM, although this is a time at which neither persistent PKC activity nor protein synthesis is required for the maintenance of long-term LFI memory. These results demonstrate that calpains function in multiple roles *in vivo* during associative memory formation.

### Keywords

associative memory; learning; Aplysia; calpain; protein kinase M; protein cleavage

## 1. Introduction

Calpains, a family of evolutionarily conserved calcium-dependent cysteine proteases, are modulatory proteases that regulate substrate function. At least 14 calpain isoforms and

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**Declaration of Interest:** The authors have no conflicts of interest.

multiple splice variants have been identified in humans (Doshi and Lynch, 2009; Franco and Huttenlocher, 2005; Paquet-Durand et al., 2007; Suzuki et al., 2004; Ueyama et al., 1998; Wu and Lynch, 2006) with calpain-1 and calpain-2 the primary isoforms found in neurons (Baudry and Bi, 2016; Baudry et al., 2013; Briz and Baudry, 2016). Calpain-1 is more sensitive to calcium with *in vitro* activation occurring at micromolar concentrations while calpain-2 requires near millimolar concentrations of calcium for activation (Baudry and Bi, 2016; Jourdi, 2014). The role of calpains in memory was suggested more than three decades ago with neuronal calpain activity postulated as critical in translating post-synaptic calcium into long-term synaptic changes following the induction of long-term potentiation (Lynch and Baudry, 1984). Post-synaptically, calpains have a wide range of targets including cytoskeletal elements, post-synaptic density proteins and glutamate receptors (Baudry et al., 2011; Dong et al., 2004; Doshi and Lynch, 2009; Vinade et al., 2001). Pharmacological inhibition of calpain activity blocks high-frequency stimulation induced LTP *in vitro* (del Cerro et al., 1990; Oliver et al., 1989). Defining the role of calpain activity in neural plasticity has been complicated as calpain-1 knockout mice display no deficits in either contextual fear conditioning or in HFS induced LTP (Grammer et al., 2005). However, the role of calpain-1 in synaptic plasticity may be mechanism dependent as conditional disruption of calpain-1 impairs LTP induced by theta burst stimulation (Zhu et al., 2015). Mice with calpain-1 deficiency in the central nervous system also demonstrate decreased performance on the last three days of an eleven day training paradigm in the Morris water maze suggesting decrements in spatial learning (Amini et al., 2013). Calpain-2 knockout mice are non-viable, but viral mediated down regulation of calpain-2 impairs LTP and Y maze alternation performance (Zadran et al., 2013). Recent *in vitro* research reveals the complexity of the role of calpains in synaptic plasticity as activation of calpain-2 limits the magnitude of theta burst induced LTP (Wang et al., 2014) and pharmacological inhibition of calpain-2 enhances high frequency stimulation induced LTP (Liu et al., 2016).

More recently, calpains have been suggested to be critical regulators for numerous brain functions including neuronal migration, neuronal differentiation, neuroprotection and synaptic plasticity (Briz and Baudry, 2016; Tan et al., 2006). Excessive or deregulated calpain activation is associated with ischemic cell death, neurodegenerative diseases including Alzheimer's disease (Cho et al., 2015), and pathological necrosis (Paquet-Durand et al., 2007). Despite the increasing number of studies investigating calpain function, questions still remain regarding the role of calpains in memory under physiological conditions. We investigated the *in vivo* role of calpain activity in intermediate and long-term associative memory.

The marine mollusk *Aplysia californica* has long been recognized as an outstanding model for examining memory due to its relatively simple nervous system and the high degree of conservation in cellular signaling mechanisms. The plasticity of feeding behaviors permit *in vivo* investigation of associative memory through appetitive and aversive learning paradigms (Hawkins and Byrne, 2015; Nargeot and Simmers, 2011; 2012). We investigated the requirements of calpain protease activity for intermediate and long-term memory formation using an associative operant learning paradigm, learning that food is inedible (LFI). For LFI memory, a single training session induces short (30 min), intermediate (4 – 6 hour) and long-term (24 hour) memory forms that are temporally and mechanistically distinct (Michel et al.,

2011a; Michel et al., 2012; Michel et al., 2011b). We found that the induction and consolidation of intermediate-term memory (ITM) required calpain activity, whereas the induction but not the molecular consolidation of long-term memory (LTM) required calpain activity. However, calpain activity was necessary during a later stage of memory maintenance, potentially involving structural remodeling associated with LTM. This study demonstrates the multiple roles of calpains *in vivo* during memory formation.

## 2. Materials and Methods

### 2.1 Animal Maintenance and Behavior Training:

Animals weighing 100–200g (Alacrity, Redondo Beach, CA; Marinus Scientific; Newport Beach, CA; South Coast Bio-Marine, San Pedro, CA) were housed in individual boxes within 100 gallon circulating seawater tanks (ASW; Instant Ocean) at 15°C on 12 h light-12 h dark cycles. Animals were fed romaine lettuce three times per week. Six days prior to behavioral experiments, animals were fed to satiation on laver seaweed and subsequently removed from appetitive stimuli. Animals received LFI training as previously described (Michel et al., 2013; Michel et al., 2012). Briefly, animals were presented with seaweed wrapped in net that cannot be swallowed but which elicits appetitive and consummatory feeding behaviors including head waving, orientation of the body toward the food, biting and swallowing attempts (Susswein et al., 1986). Memory formation occurs as the animals associate a specific seaweed with the failure of the swallowing attempts resulting in decreased response times upon subsequent presentation of the netted seaweed. Training was stopped after 25 minutes by gently removing the seaweed bag during a protraction cycle by the animal. Testing was performed using the same procedures and continued until three min elapsed without the animal's intake of the seaweed bag into the mouth. Memory was represented as a decrease in two metrics, the total response time and the time the food was retained in the mouth, compared to the responses of naïve animals. As intermediate and long-term LFI memories are strongly modulated by the circadian clock (Lyons et al., 2005; Michel et al., 2013), all training was performed at Zeitgeber Time 3 (three hours after lights on) to eliminate any variation in memory formation.

### 2.2 Drug Treatments:

Animals were injected through the anterior portion of the foot with either inhibitor or vehicle. As previously, systemic drug calculations were projected based upon the percentage of the body-weight comprised by the hemolymph, approximated at 65% of total body weight (Levenson et al., 1999; Michel et al., 2013). Calpeptin (EMD Millipore Calbiochem) is a cell-permeable specific calpain inhibitor previously used in *Aplysia* neuronal cell cultures (Khoutorsky and Spira, 2005; 2009) that acts via calpain's active site (Figueiredo-Pereira et al., 1994; Tsujinaka et al., 1988). Calpeptin was used at an approximated systemic concentration of 10 μM prepared as a 10 mM DMSO stock solution, diluted to 0.65 mM working stock with ASW, and animals injected with 1 ml/100 gram body weight of the working stock solution (final DMSO concentration was 65 μl per ml). Vehicle injected animals were injected with 1 ml/100 gram body weight of DMSO diluted in ASW in the same ratio as for calpeptin (65 μl DMSO per ml injected solution). MDL-28170 (Enzo), also known as Calpain Inhibitor III, is a reversible, membrane permeant calpain-specific inhibitor

(Mehdi, 1991) that has previously been used with *Aplysia* semi-intact preparations (Sutton et al., 2004) and in mammals for studies of memory and synaptic plasticity (Briz et al., 2013; Yu et al., 2011; Zadran et al., 2013). MDL-28170 was prepared as 5mM DMSO solution and injected at 130 μl/100 gram animal body weight for an approximate systemic concentration of 10 μM. DMSO (130 μl/100 gram animal body weight) was injected as the vehicle in control animals. No differences were observed in the behavioral responses of vehicle injected and non-injected animals upon presentation of netted seaweed. Treated animals were only used in a single experiment.

### 2.3 Statistics:

Statistical analysis of the data was carried out using one-way ANOVA with Bonferroni post-hoc analyses. *P* values less than 0.05 were considered significant.

## 3. Results

### 3.1 Calpain activity is necessary for the induction of intermediate-term memory

In *Aplysia*, multiple isoforms of protein kinase C (PKC) or the persistently active PKM forms are required for the induction of intermediate-term LFI memory (Michel et al., 2012). Generation of the constitutively active PKM isoforms including the classical PKM Apl I and the atypical PKM Apl III arises through calpain dependent protein cleavage (Bougie et al., 2009; Hastings et al., 2013; Sutton et al., 2004). Calpain activity is necessary for the generation of PKMs during the induction and maintenance of non-associative site-specific intermediate-term sensitization in *Aplysia* (Sutton et al., 2004). Calpain activity also is necessary for serotonin-induced generation of PKM Apl III during facilitation (Bougie et al., 2012; Bougie et al., 2009; Villareal et al., 2009). We investigated whether calpain activity was required for the induction of operant memory using calpeptin, a peptide aldehyde cell-permeable inhibitor frequently used *in vivo* (Mani et al., 2008; Samantaray et al., 2015). Animals were injected with calpeptin 30 min prior to training and tested 4 h after training. Calpeptin injected animals failed to display ITM with responses comparable to treated animals that did not receive training, while control animals showed significantly reduced response times during testing compared to naïve animals (Figure 1A and 1B). Animals injected with calpeptin that received no LFI training demonstrated behavioral responses during testing similar to naïve animals indicating that the drug alone had no effect on appetitive or consummatory baseline feeding responses. Although these results suggest that calpain inhibition impedes the formation of memory, calpeptin may also inhibit other proteases including cathepsins and the proteasome. Recent research has demonstrated that proteasome inhibition using a low dose of MG-132 does not block the induction of intermediate-term LFI memory (Lyons et al., 2016), suggesting that calpeptin does not obstruct memory via proteasomal inhibition. However, a higher concentration of MG-132 sufficient to inhibit proteasome and calpain activity does inhibit intermediate-term LFI memory (Supplemental Data Figure 1) suggesting that calpain activity is necessary for the induction of ITM.

To confirm the calpeptin experiments and to control for possible off-target inhibitor effects, we used another calpain inhibitor MDL-28170 that has previously been used in *Aplysia* and

rodent models for investigating the role of calpains in memory and synaptic plasticity (Briz et al., 2013; Shimizu et al., 2007; Sutton et al., 2004; Yu et al., 2011; Zadran et al., 2013). Animals were injected with MDL-28170 thirty minutes prior to training and tested four hours after training. As with calpeptin, animals injected with MDL-28170 prior to training demonstrated no ITM with response times similar to naïve animals or animals injected with MDL-28170 that did not receive training (Figure 1C and 1D). Vehicle-injected animals exhibited robust ITM with significantly decreased response times compared to naïve animals. The drug alone did not significantly change baseline responses from those observed in naïve animals. While these experiments do not identify the specific calpain isoforms necessary for the induction of ITM, these results suggest that calpain-dependent protein cleavage is an important component of LFI memory formation.

### 3.2 Maintenance of intermediate-term memory requires calpain activity

Intermediate-term LFI memory requires PKM Apl III activity throughout the duration in which memory is maintained (Michel et al., 2012). If calpain cleavage is necessary for generation of persistent PKC forms, then calpain activity may also be required after ITM training. To test this hypothesis, animals were injected with calpeptin immediately following LFI training and tested four h later. Inhibition of calpain activity after training blocked ITM with no significant decreases observed in either total response time or the time the seaweed was retained in the mouth for treated animals (Figure 1E and 1F). In contrast, vehicle-injected controls displayed robust ITM with response times significantly decreased compared to naïve animals. Thus, calpain activity appears necessary for the induction and maintenance of ITM.

### 3.3 Calpain activity is necessary for the induction of long-term memory

Long-term LFI memory also seems to require activity of multiple isoforms of PKC for the induction of memory, but not for the maintenance or recall of memory (Michel et al., 2012; Michel et al., 2011b). To determine whether LFI memory requires calpain activity for the induction of LTM, animals were injected with calpeptin 30 min prior to training and tested 24 h later. We found that vehicle-treated animals displayed robust LTM while calpeptin-treated animals displayed no LTM with responses similar to naïve animals (Figure 2A and 2B). Calpeptin injection alone in the absence of training had no effect on the behavioral responses of the animal to seaweed 24 h after drug injection with observed response times similar to naïve animals. We performed similar experiments using MDL-28170. As with calpeptin, injection of MDL-28170 prior to training inhibited LTM when animals were tested 24 h later with treated animals exhibiting response times similar in length to animals that received the drug but no training (Figure 2C and 2D). Vehicle-treated animals demonstrated LTM with significantly reduced response times compared to naïve animals. These results suggest that calpain activity is required for the induction of LTM.

### 3.4 Maintenance of LTM, but not the molecular consolidation of memory, requires calpain activity

Although PKMs do not appear to be required for the maintenance of long-term LFI (Michel et al., 2012; Michel et al., 2011b), calpains potentially play multiple roles in memory independent of PKM generation. For longer forms of memory, calpains may function in

synaptic remodeling through proteolytic cleavage of cytoskeletal elements (Briz and Baudry, 2016; Jourdi, 2014). It has also been hypothesized that calpain activity affects dendritic protein synthesis associated with synaptic plasticity (Briz and Baudry, 2016; Briz et al., 2013). In our preceding experiments, although both calpeptin and MDL-28170 are reversible *in vivo* these inhibitors likely retain some efficacy during the molecular consolidation phase of LFI memory even though the inhibitors were injected prior to training. For 24 h LFI memory, the window of molecular consolidation and macromolecular synthesis spans the first few hours after training with protein synthesis no longer required six hours post-training (Levitan et al., 2008; Levitan et al., 2010). To specifically test the requirement for calpain activity during the periods of molecular consolidation and maintenance of LTM, we injected the calpain inhibitors following LFI training. Animals were injected with calpeptin immediately after training and tested for LTM 24 hours later. Animals treated with calpeptin immediately after training displayed robust LTM with similar response times to vehicle-treated animals (Figure 3A and 3B). These results suggest that calpain activity is necessary for the induction of LTM, but not the molecular consolidation necessary for memory formation.

In addition to macromolecular synthesis, long-term memory may involve synaptic remodeling or the growth of new synapses for the maintenance of memory (Bailey et al., 1992; Bailey and Kandel, 2008). To determine whether later processes necessary for the maintenance of LTM required calpain activity, we injected animals with calpeptin six hours after LFI training. Interestingly, the late injection of calpeptin completely blocked LTM at 24 hours (Figure 3C and 3D) with animals displaying response times similar to naïve animals. Vehicle-treated animals displayed robust LTM. Similarly, animals injected with MDL-28170 six hours after training demonstrated no memory 24 h later with response times similar to naïve animals (Figure 3C and 3D). These results suggest that calpain dependent protein cleavage is necessary for later processes in memory such as synaptic remodeling or strengthening necessary for the long-term maintenance of memory.

## 4. Discussion

Recently, there has been resurgent interest in delineating the function of protein cleavage and protein degradation in synaptic plasticity. In the current study, we investigated the *in vivo* role of calpains in intermediate and long-term associative memory using *Aplysia* because of the amenability of the system for behavioral pharmacology studies. Invertebrates are frequently referred to as simple model systems due to the small number of neurons in their central nervous systems; however, these models display complexity in signaling pathways comparable to vertebrate systems. Associative learning paradigms exploiting the highly plastic *Aplysia* feeding system have revealed the intricacies of memory formation and its modulation (Elliott and Susswein, 2002; Hawkins and Byrne, 2015; Michel and Lyons, 2014; Nargeot and Simmers, 2011). The LFI paradigm is an ethologically relevant learning paradigm, for which a single training session induces temporally and mechanistically distinct forms of memory allowing direct comparisons of the underlying mechanisms involved in memory formation (Michel et al., 2012).

We found that inhibition of calpain activity prior to LFI training blocked the induction of both intermediate and long-term memory. Calpain activity is necessary for the generation of multiple persistently active PKC forms including PKM Apl I and PKM Apl III (Hastings et al., 2015; Hastings et al., 2013). As both intermediate and long-term LFI memory appear to require multiple forms of PKC for the induction of memory, calpain activation of PKM isoforms pre-or post-synaptically may provide cellular specificity. Calpains localize pre-and post-synaptically (Hastings et al., 2015; Hastings et al., 2013) with potential roles for both presynaptic and postsynaptic calpain activity in the induction of memory. Pre-synaptically, facilitation of depressed synapses in cultured neurons through PKC-induced mobilization of synaptic vesicles requires calpain activity (Khoutorsky and Spira, 2005). Furthermore, inhibition of calpain in cultured *Aplysia* neurons transforms a facilitating synapse into a depressed one (Khoutorsky and Spira, 2009). In long-term facilitation, the atypical PKC (Apl III) undergoes calpain dependent cleavage to form PKM in the post-synaptic neuron (Cai et al., 2011; Villareal et al., 2009) raising the possibility of post-synaptic calpain activity for LFI memory. In mice, post-synaptic calpain activity may be necessary for the regulation of glutamate receptors on dendritic spines as multiple glutamate receptors and their scaffolding proteins are calpain targets (Baudry et al., 2013; Jourdi, 2014; Lynch and Baudry, 1984), even though baseline glutamate neurotransmission does not appear altered in mutant mice (Amini et al., 2013). Thus, the possibilities exist that during the induction of LFI memory, calpains may be necessary for PKC mediated mobilization of synaptic vesicles or the post-synaptic enhancement of neurotransmission. Alternatively, calpain activity may augment common signaling pathways necessary for the induction of intermediate and long-term LFI memory such as MAPK signaling. In mouse hippocampal slices, inhibition of calpain activity blocks BDNF mediated increases in MAPK signaling (phospo-ERK; (Wang et al., 2014).

It should be noted that the calpain inhibitors we used, calpeptin and MDL-28170, display some cross-reactivity with other proteases most notably cathepsins. Cathepsins are lysosomal proteases whose activity has been associated with inflammation, autophagy, apopotosis and neurodegeneration (Stoka et al., 2016) and for which inhibition has been suggested as therapeutic to enhance memory (Hook et al., 2011; Kindy et al., 2012). In general, cathepsin activity appears to be associated with decrements in learning and memory. For example, *Drosophila* mutants with a mutation in a gene encoding an inhibitor of cathepsins exhibit LTM deficits (Comas et al., 2004). Thus, we think it is more likely that the inhibition of memory we observed was through inhibition of calpain activity rather than cathepsin inhibition.

In addition to the early role of calpain in memory, we found that intermediate-term memory required calpain activity post-training. Intermediate-term LFI memory requires the activity of multiple kinase pathways, including PKM Apl III, during training and throughout the entire timeframe the memory perseveres (Michel et al., 2012). Unlike in mammals, persistent forms of PKC in *Aplysia* appear to arise solely from calpain-mediated protein cleavage (Bougie et al., 2012; Bougie et al., 2009; Villareal et al., 2009). Non-associative forms of ITM including site-specific sensitization and facilitation require calpain-dependent proteolysis for the generation of the persistently active PKC isoforms (Bougie et al., 2012; Sutton et al., 2004). Although recent research suggests that *Aplysia* have multiple calpains

with distinct roles in the generation of PKM from different PKC isoforms dependent upon neuron type and conditions (Hastings et al., 2015), our experiments cannot distinguish the calpain isoform(s) that function in LFI memory as isoform specific calpain inhibitors are not available in *Aplysia*. The requirement for prolonged calpain activity in ITM is consistent with the role of a classical calpain in generation of PKM Apl III. However, we cannot rule out that calpain activity also may be necessary for protein synthesis during ITM formation. In multiple *in vitro* mammalian preparations, calpain-2 protein cleavage promotes BDNF stimulated dendritic protein synthesis (Briz et al., 2013).

In contrast to the requirement for calpain activity post-training in ITM, we found that calpain activity was not required during the time frame of molecular consolidation in LTM. If the primary role of calpain during this time period is the generation of PKM then these results are consistent with previous research as persistently active forms of PKC are not required post-training for long-term LFI memory (Michel et al., 2012; Michel et al., 2011b). These results also suggest that calpain activity is unnecessary for protein synthesis during the molecular consolidation of LTM. However, we found that calpain activity was required 6 h after training to maintain LTM at 24 h. As protein synthesis is not required during this time frame for 24 h LFI memory (Levitan et al., 2010), we hypothesize that calpain activity 6 h post-training targets cytoskeletal proteins permitting synaptic expansion or the growth of new synapses. In mammals, dendritic proteins involved in actin cytoskeletal dynamics including spectrin, RhoA, Drebin, MARCSK are cleaved by calpains facilitating rearrangement of actin cytoskeleton networks and permitting synaptic growth (Baudry and Bi, 2016; Baudry et al., 2011; Baudry et al., 2013). Although long-term memory storage and synaptic growth may be dissociated as recently shown in *Aplysia* (Chen et al., 2014), synaptic remodeling and the growth of new synapses is considered an integral part of long-term memory stability (Bailey and Kandel, 2008; Kandel, 2012).

Overall, our behavioral studies suggest that while calpains may have a common role in the induction of LFI memory, the role of calpain dependent cleavage appears to differ considerably for the consolidation and maintenance of ITM and LTM. The temporal distinctions in the requirement for calpain activity between ITM and LTM also suggest differences in calpain function and target substrates for these two forms of memory raising questions for future studies investigating the physiological roles of calpains in memory formation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

We wish to thank Chelsea Organ for assistance with animal maintenance and preliminary behavioral experiments. This research was supported by grants from the National Institute of Mental Health grant R01MH81012 and National Institute of Neurological Disorders and Stroke grant R21NS088835.

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## Highlights

- Inhibition of calpain activity with calpeptin or MDL-28170 prevented ITM formation.
- Calpain inhibition prior to training blocked the induction of LTM.
- Inhibiting calpain activity immediately after training impaired ITM but not LTM.
- Calpain activity at 6 h post-training is necessary for maintenance of 24 h LTM.
- Calpains function in multiple roles in associative memory formation.

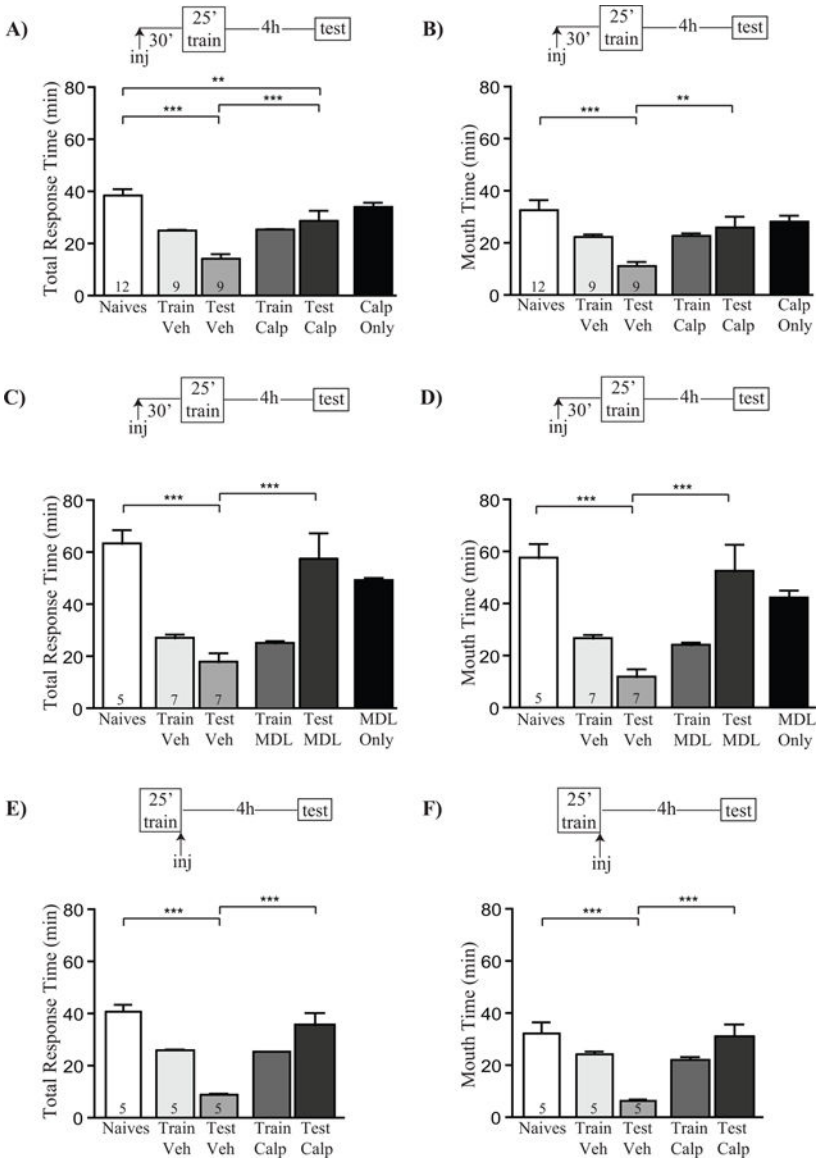


**Figure 1: Induction and maintenance of ITM requires calpain activity.**

**(**A) Calpeptin injection (~10μM systemic concentration) prior to training blocked ITM tested 4 hours after training, while vehicle-injected animals exhibited significantly decreased response times as observed for (A) total response time, (ANOVA $F_{(5, 45)} = 15.79$, $p < 0.0001$) and (B) total mouth time, (ANOVA $F_{(5, 45)} = 6.77$, $p < 0.0001$). Calpeptin alone in the absence of training had no effect on baseline behavior as drug only animals displayed responses comparable to naïve animals. Means and standard error of the mean plotted for all experiments. Post-hoc analyses performed using Bonferroni corrections for multiple comparisons with significance as $^{**}p < 0.01$ and $^{***}p < 0.001$ designated with asterisks. (C and D) MDL-28170 injection (~10μM systemic concentration) prior to training blocked ITM with drug injected animals exhibiting response times similar to naïve animals and drug-treated animals that did not receive training. Vehicle (DMSO) injected animals displayed robust memory with significantly decreased response times for (C) total response time,

(ANOVA $F_{(5, 31)} = 12.86$, $p < 0.0001$) and (D) total mouth time, (ANOVA $F_{(5, 31)} = 11.29$, $p < 0.0001$). MDL-28170 alone had no effect on baseline behavior as drug only animals displayed responses comparable to naïve animals. (E and F) To determine if sustained calpain activity was necessary for ITM, animals were injected with calpeptin immediately after LFI training. Post-training calpeptin inhibited ITM while vehicle-injected animals exhibited robust ITM (E) total response time, (ANOVA $F_{(4, 24)} = 18.68$, $p < 0.0001$) and (F) total mouth time, (ANOVA $F_{(4, 24)} = 10.56$, $p < 0.0001$).

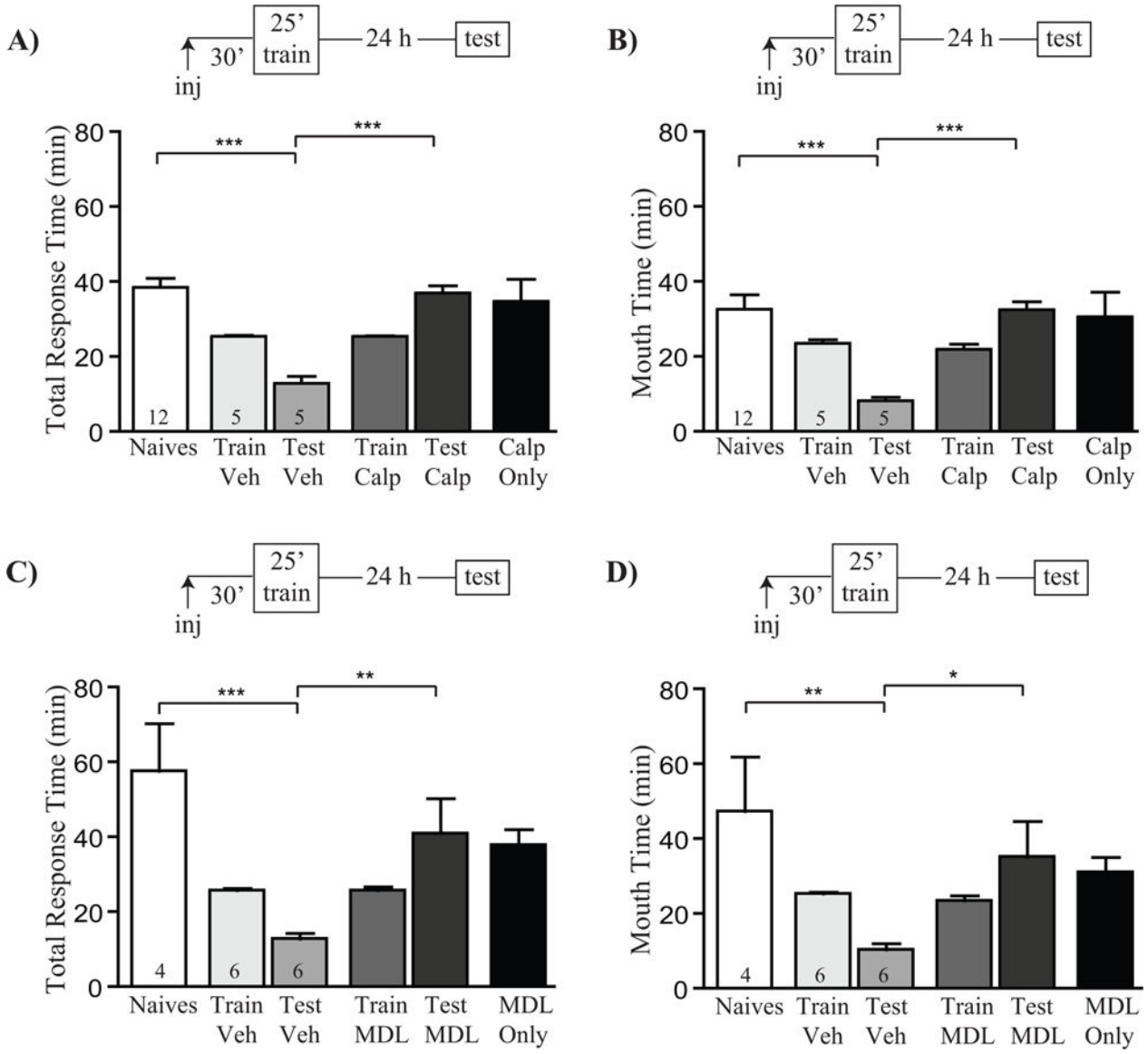


**Figure 2: Long-term memory requires calpain activity during induction.**

Calpeptin treatment 30 min before training blocked LTM whereas vehicle-injected controls showed significant LTM; (A) total response time, (ANOVA $F_{(5, 39)} = 14.04$, $p < 0.0001$), (B) total mouth time, (ANOVA $F_{(5, 39)} = 6.423$, $p < 0.0001$). Calpeptin alone in the absence of training had no effect on baseline behavior as drug only animals displayed responses comparable to naïve animals. Asterisks denote significant differences with ***$p < 0.001$. (C and D) Animals were injected with MDL-28170 thirty min prior to training and tested 24 h later. Drug treated animals failed to show LTM with response times similar to drug treated animals that were not trained. Vehicle-injected animals exhibited robust LTM for (C) total response time, (ANOVA $F_{(5, 30)} = 5.883$, $p < 0.001$) and (D) total mouth time, (ANOVA $F_{(5, 30)} = 3.479$, $p < 0.01$). MDL-28170 alone had no effect on behavior as the responses of drug only animals were not significantly different than naïve animals. Asterisks denote significant differences with *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

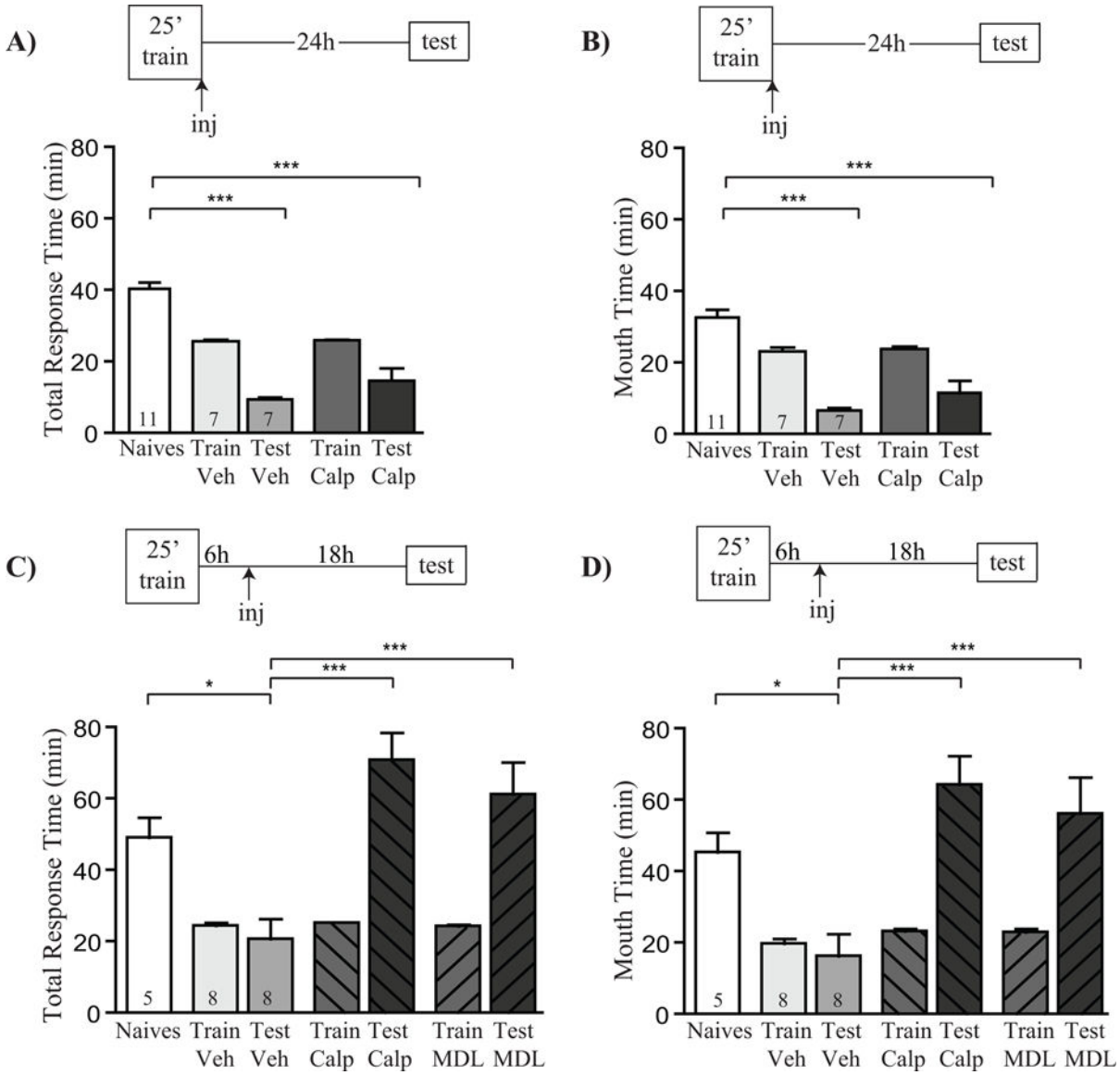


**Figure 3: Calpain activity is required for the maintenance of long-term memory, but not memory consolidation**

Calpeptin injected immediately after training did not affect LTM assessed 24 h later with significantly decreased response times observed for treated and vehicle-injected animals in (A) total response time, (ANOVA $F_{(4, 38)} = 42.86$, $p < 0.0001$) and (B) total mouth time, (ANOVA $F_{(4, 38)} = 25.25$, $p < 0.0001$). (C) To determine if calpain activity was necessary at stages following the period of macromolecular synthesis for 24 hour memory, calpain inhibitors (calpeptin and MDL-28170) were injected 6 hours post-training. Inhibition of calpain activity blocked the maintenance of LTM while vehicle-injected animals exhibited significantly decreased response times as compared to naïve animals for (C) total response time, (ANOVA $F_{(6,48)} = 15.49$, $p<0.0001$) and (D) total mouth time, (ANOVA $F_{(6,48)} = 11.31$, $p<0.0001$). Asterisks denote significant differences with ***$p < 0.001$, **$p<0.01$ and *$p<0.05$.