Brief Communication

PKA and PKC are required for long-term but not short-term in vivo operant memory in *Aplysia*

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We investigated the involvement of PKA and PKC signaling in a negatively reinforced operant learning paradigm in *Aplysia*, learning that food is inedible (LFI). In vivo injection of PKA or PKC inhibitors blocked long-term LFI memory formation. Moreover, a persistent phase of PKA activity, although not PKC activity, was necessary for long-term memory. Surprisingly, neither PKA nor PKC activity was required for associative short-term LFI memory. Additionally, PKA and PKC were not required for the retrieval of short- or long-term memory (STM and LTM, respectively). These studies have identified key differences between the mechanisms underlying nonassociative sensitization, operant reward learning, and LFI memory in *Aplysia*.

[Supplemental material is available for this article.]

Signaling cascades and the mechanisms underlying memory appear highly conserved across phylogeny with similar mechanisms identified in vertebrates and invertebrates (Sweatt 2003). Studies using the marine mollusk *Aplysia* have been critical in providing fundamental knowledge regarding the molecular basis of memory (for review, see Bailey et al. 2008). The cAMP-PKA signaling pathway represents a classical pathway necessary for many types of memory and synaptic plasticity, including presynaptic facilitation in *Aplysia* (for review, see Kandel 2001), olfactory conditioning in *Drosophila* (for review, see Davis 2005), long-term associative memory in mammals (for review, see Selcher et al. 2002), and late long-term potentiation (LTP) (Abel et al. 1997; Huang et al. 2000). Similarly, PKC signal transduction has been shown as integral for memory in *Aplysia* (Sacktor et al. 1988; Sugita et al. 1992; Sossin et al. 1994; Manseau et al. 1998), LTP (for review, see MacDonald et al. 2001; Sacktor 2008), and contextual fear conditioning (Atkins et al. 1998). Considerable intersection exists between the PKA and PKC signaling pathways during memory formation (Sugita et al. 1997; Lorenzetti et al. 2008). Despite the extensive research in *Aplysia*, few studies have been done on signaling in vivo due to limitations in genetic tractability and available species-specific reagents. In the present research, we have compared the roles of PKA and PKC between short-term memory (STM) and long-term memory (LTM) using in vivo behavioral studies.

We examined the role of PKA and PKC using the operant conditioning paradigm, learning that food is inedible (LFI). In this paradigm, animals associate a netted seaweed with the failure to swallow resulting in memory that the food is inedible. As previously described (Lyons et al. 2005, 2006), *Aplysia californica* were fed to satiation with laver seaweed 5 d prior to an experiment. Individual animals were trained using netted seaweed to which animals responded with repeated cycles of biting, swallowing attempts, and rejection of the food. Memory depends upon swallowing attempts followed by signaling from the gut denoting the failure of feeding (Schwarz and Susswein 1986; Schwarz et al. 1991). Presentation of the netted seaweed continued until the

animal rejected the food for 3 min without re-entry into the mouth. Upon testing, memory was measured as a significant reduction in the total response time and the time seaweed was retained in the mouth compared to initial training. A single training session results in associative LTM specific for the seaweed used during training (Susswein et al. 1986). To confirm that 30 min STM also represents a specific association with the seaweed from training, we trained one set of animals with laver and a second set with kombu seaweed. During testing, half the animals were tested with the seaweed from training while the other half was tested with the alternate seaweed. STM was observed only in animals for which the same seaweed was used for training and testing (Supplemental Fig. 1). Animals in which testing occurred using the alternate seaweed exhibited responses similar to training times. As with LTM, short-term LFI memory represents specific learned associations rather than a broader decrement in feeding responses. Since STM and LTM are also temporally distinct (Botzer et al. 1998), the LFI paradigm provides the opportunity to distinguish between signaling pathways recruited for LTM and those necessary for STM.

In *Aplysia*, PKA is necessary for multiple types of LTM including sensitization, classical conditioning, and operant learning (for review, see Baxter and Byrne 2006; Bailey et al. 2008). To investigate the role of PKA in operant memory in vivo, animals were injected 30 min before training with vehicle (ASW) or 1 mL/100 g body weight of 19.5 μ M H89 (Sigma), a PKA inhibitor previously used in *Aplysia* (Farah et al. 2009) that binds to the catalytic subunit (Hidaka et al. 1984, 1990). This yielded a predicted 300-nM H89 systemic concentration estimated based on hemolymph representing 65% of body weight (Levenson et al. 1999). In all experiments, animals were trained at Zeitgeber time 3 to eliminate circadian variance. No significant difference was observed in training responses between vehicle-injected and inhibitor-treated animals (Supplemental Fig. 2A,B). Upon testing 24 h later, vehicle-injected animals demonstrated robust memory with significantly reduced response times, while animals treated with H89 exhibited no LTM (Fig. 1A). Similar results were observed for a second parameter used to assess memory, the time the seaweed was retained in the mouth (Supplemental Fig. 3). These results suggest that LTM requires PKA activity. However, H89 has been reported to exhibit broader effects on

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Figure 1. Prolonged PKA activity is necessary for long-term (LTM) but not short-term memory (STM). (A) Inhibition of PKA with 300-nM H89 30 min before training prevented LTM. Vehicle-injected animals displayed significantly decreased response times (control $n = 14$, H89 $n = 15$; $F_{(3,57)} = 8.389$, $P < 0.001$). Mean and SEM are plotted. (B) Rp-cAMPS injected before training blocked LTM (control $n = 5$, Rp-cAMPS $n = 6$; $F_{(3,21)} = 6.335$; $P < 0.05$). (C) Prolonged PKA is required for LTM. H89 injected 5 min after training inhibited LTM, while vehicle-injected animals exhibited significant LTM (control $n = 9$, H89 $n = 8$; $F_{(3,33)} = 6.588$, $P < 0.01$). (D) H89 had no effect on STM formation as both control and drug-treated animals demonstrated significant STM (control $n = 14$, H89 $n = 12$; $F_{(3,51)} = 34.56$, $P < 0.001$). (E) Injection of 10 μ M Rp-cAMPS did not block STM (control $n = 5$, Rp-cAMPS $n = 6$; $F_{(3,21)} = 18.97$, $P < 0.001$). Data analysis was performed using ANOVA followed by Bonferroni's multiple comparison test (MCT). Asterisks denote significant differences with $P < 0.05$ for the testing vs. training groups. White bars represent mean response times for vehicle-treated animals, while gray bars signify mean response times for drug-treated animals.

mitogen and stress-activated protein kinase 1, Rho-dependent protein kinase II, and p70 ribosomal protein S6 kinase (Davies et al. 2000). Potentially, interference with one of these kinases could result in inhibition of LFI memory as all have been at least peripherally implicated in memory in other paradigms (Udo et al. 2005; Chwang et al. 2007; Sindreu et al. 2007).

To independently test the role of PKA, we used Rp-cAMPS, a phosphodiesterase resistant competitive inhibitor (de Wit et al. 1982; Rothmel et al. 1983) previously used in *Aplysia* (Ghirardi et al. 1992; Dyer and Sossin 2000; Lorenzetti et al. 2008). Injections of 1 mL/100 g of 650 μ M Rp-cAMPS (Sigma; predicted systemic concentration 10 μ M) 30 min before training significantly inhibited LTM compared to vehicle-injected animals (Fig. 1B; Supplemental Fig. 3B). This lack of LTM upon treatment with either Rp-cAMPS or H89 prior to training strongly suggests a requirement for PKA.

It remained possible that the long responses observed during testing were due to persistent effects of the drugs in vivo that affected feeding behavior through a mechanism independent of LTM. To test this possibility, animals were injected with vehicle, H89, or Rp-cAMPS and tested 24 h later. No significant differences in response times were found between vehicle and inhibitor-injected animals indicating that the inhibitors alone do not result in increased appetitive or consummatory behavioral responses (Total Time [min]: controls 37.8 ± 3.3 [$n = 11$], H89 32.0 ± 3.0 [$n = 6$], Rp-cAMPS 30.0 ± 6.0 [$n = 6$], ANOVA $P = 0.35$; Time in the Mouth [min]: controls 36.8 ± 3.1 ; H89 29.1 ± 3.0 ; Rp-cAMPS 25.9 ± 6.0 , ANOVA $P = 0.14$).

In many forms of learning, PKA activation is necessary for the induction of LTM and the molecular consolidation of memory (Bailey et al. 2008). During long-term facilitation, a prolonged requirement for PKA activity lasting several hours appears necessary for LTM (Müller and Carew 1998). To determine whether long-term LFI required persistent PKA activity, we injected animals with H89 5 min after training targeting a time when the

Figure 2. PKC is required for the induction of LTM but not STM. (A) Inhibition of PKC before training with chelerythrine inhibited LTM. Vehicle-injected animals exhibited robust memory with significantly decreased response times (control $n = 15$, chelerythrine $n = 15$; $F_{(3,59)} = 22.66$; $P < 0.001$). (B) Bisindolylmaleimide I (control $n = 14$, bis $n = 13$; $F_{(3,53)} = 4.738$; $P < 0.01$) treatment before training also blocked LTM. (C) Inhibition of PKC with chelerythrine after training had no effect on LTM (control $n = 9$, chelerythrine $n = 8$; $F_{(3,33)} = 12.14$, $P < 0.001$). (D) The PKC inhibitor chelerythrine had no effect on STM formation as treated animals displayed robust STM comparable to controls (control $n = 13$, chelerythrine $n = 10$; $F_{(3,45)} = 37.86$, $P < 0.001$). (E) Bisindolylmaleimide I failed to inhibit STM formation (control $n = 7$, bis $n = 8$; $F_{(3,29)} = 33.78$; $P < 0.001$). Data analysis was performed using ANOVA followed by Bonferroni's MCT. Asterisks denote significant differences with $P < 0.05$ for the testing vs. training groups.

Figure 3. PKA and PKC are not required for the recall of memory. (A) Animals were injected with either vehicle (ASW), H89, or chelerythrine immediately after training and then tested for STM 30 min later. Neither the inhibitor for PKA nor the inhibitor of PKC blocked the expression or retrieval of STM with all groups exhibiting comparable significant STM ($n = 6$ for each group; $F_{(5,35)} = 20.07$; $P < 0.001$). (B) Animals were trained and 24 h later injected with either vehicle (ASW), H89, or chelerythrine 30 min prior to testing. Neither the inhibitor for PKA nor the inhibitor of PKC blocked the recall of LTM with all groups exhibiting comparable significant LTM ($n = 9, 8, 9$, respectively; $F_{(5,51)} = 40.86$; $P < 0.001$). Data analysis was performed using ANOVA followed by Bonferroni's MCT. Asterisks denote significant differences with $P < 0.05$ for the testing vs. training groups.

induction of memory had already been initiated (Botzer et al. 1998). Control animals exhibited robust LTM, while animals treated with H89 after training demonstrated no LTM with response times similar to training times (Fig. 1C). This requirement for prolonged PKA in LFI is consistent with previous research as PKA performs a vital role in the activation of CREB-induced transcription (for review, see Hawkins et al. 2006; Bailey et al. 2008).

In *Aplysia*, PKA appears necessary for most short-term plasticity including facilitation (Byrne and Kandel 1996), associative plasticity (Ocorr et al. 1985; Abrams et al. 1998), and in vitro operant reward learning (Lorenzetti et al. 2008). However, when a broader spectrum of paradigms is examined across species, the role of PKA in STM appears more variable. PKA is required for LTM but not STM in fear conditioning (Abel et al. 1997; Goossens et al. 2000; Schafe and LeDoux 2000) and conditioned taste aversion (Koh et al. 2002). As many of the studies establishing PKA in STM in *Aplysia* occurred in vitro, we investigated PKA in short-term LFI. Animals were injected with vehicle or inhibitor 30 min before training and tested for STM 30 min later. Surprisingly, H89 had no effect on STM as treated animals displayed significant memory similar to controls (Fig. 1D; Supplemental Fig. 3D). Potentially, the lack of H89 effect was concentration dependent, although the dosage was sufficient to block LTM and exceeded the reported IC_{50} (Davies et al. 2000). To further probe the cAMP-PKA pathway in STM, we performed experiments with Rp-cAMPs as described above. Injection of Rp-cAMPs had no effect on STM as treated animals displayed significant memory comparable to controls (Fig. 1E). To verify that results were not due to inhibitor-induced decreases in feeding responses,

we assessed the effect of H89 and Rp-cAMPs 90 min after injection. Inhibitors alone did not significantly affect response times (Total Response Time [min]: controls 29.6 ± 3.2 [$n = 17$], H89 24.6 ± 3.0 [$n = 7$], Rp-cAMPs 28.5 ± 4.5 [$n = 4$], ANOVA $P = 0.64$; Time in Mouth [min]: controls 26.0 ± 3.6 ; H89 21.7 ± 3.1 ; Rp-cAMPs 25.0 ± 3.4 , ANOVA $P = 0.76$). In contrast to short-term facilitation and neuronal excitability, these results strongly suggest that PKA is not necessary for induction or formation of short-term LFI memory.

For the in vitro analog of operant reward learning, a calcium-mediated increase in PKC activity is proposed to modulate the PKA cascade (Baxter and Byrne 2006; Lorenzetti et al. 2008), suggesting the potential of PKC involvement in negatively reinforced operant memory. PKC also has been implicated in long-term facilitation (Sossin et al. 1994; Manseau et al. 1998) and activity-dependent facilitation (Hu et al. 2007). To test the involvement of PKC in long-term LFI, animals were injected with the PKC inhibitor chelerythrine, which inhibits all classes of PKC (Herbert et al. 1990; Sossin 2007) and was previously used in many *Aplysia* studies (Khabour et al. 2004; Bougie et al. 2009; Villareal et al. 2009). We injected vehicle (ASW) or 1 mL/100 g of 43.55 μ M chelerythrine (Calbiochem; 670 nM predicted systemic concentration) 30 min before training and tested animals 24 h later. Inhibition of PKC with chelerythrine abolished LTM, while vehicle-injected animals exhibited robust memory (Fig. 2A). Similar results were observed for the time the food was retained in the mouth (Supplemental Fig. 4). Chelerythrine inhibits PKC via the catalytic domain (Herbert et al. 1990), so we investigated LTM using an inhibitor with a different mechanism of action, Bisindolylmaleimide I (Bis, Calbiochem), which acts at the ATP binding site (Toullec et al. 1991) and also was previously used in *Aplysia* (Kabir et al. 2001; Lim and Sossin 2006; Villareal et al. 2009). Injection of animals with 130 μ L/100 g 5 mM Bis (10 μ M predicted systemic concentration) 30 min prior to training completely blocked LTM, while control animals injected with DMSO demonstrated significant LTM (Fig. 2B). Neither PKC inhibitor significantly affected the responses of the animals during training (Supplemental Fig. 2C,D). While chelerythrine broadly inhibits PKC, the inhibition of LTM by Bis implicates either classical or novel PKC since the atypical isoform is not inhibited by Bis (Sossin 2007; Villareal et al. 2009). To determine if the inhibitors alone impacted response times 24 h after treatment, animals were injected with the vehicle, chelerythrine, or Bis and tested 24 h later. There was no significant difference between the responses of vehicle- or inhibitor-injected animals (Total Response Time [min]: controls 37.8 ± 3.3 [$n = 11$], chelerythrine 29.1 ± 4.8 [$n = 6$], Bis 37.5 ± 7.0 [$n = 6$], ANOVA $P = 0.39$; Time in the Mouth [min]: controls 36.8 ± 3.1 ; chelerythrine 26.6 ± 5.4 ; Bis 32.5 ± 7.2 , ANOVA $P = 0.34$).

Persistent PKC activation is necessary for the maintenance of LTP (Klann et al. 1991; Sacktor et al. 1993) and for maintenance of long-term memory in rats (Jerusalinsky et al. 1994; Shema et al. 2007; Serrano et al. 2008). In *Aplysia*, persistently active PKC is involved in long-term facilitation (Sossin et al. 1994). Persistent, autonomous forms of PKC arise by proteolytic cleavage of the regulatory domain forming PKM (Inoue et al. 1977; Sutton et al. 2004; Bougie et al. 2009), an isoform inhibited by chelerythrine (Sossin 2007; Villareal et al. 2009). To test the requirement for persistent PKC in long-term LFI, we trained animals and injected them with chelerythrine after training. Contrary to the results we observed with inhibition of PKA, post-training PKC inhibitor injection did not block LTM (Fig. 2C). Thus, we hypothesize that the role for PKC in this form of operant LTM may be upstream of cAMP-PKA signaling and enhance PKA activation as proposed for operant reward learning (Baxter and Byrne 2006; Lorenzetti et al. 2008).

In *Aplysia*, PKC has been implicated in short-term plasticity (Braha et al. 1990; Sacktor and Schwartz 1990; Sugita et al. 1992; for review, see Hawkins et al. 2006; Sossin 2007). We tested the role of PKC in STM using both chelerythrine and Bis as above. Neither chelerythrine nor Bis affected STM as treated and control groups exhibited robust memory (Fig. 2D,E), strongly suggesting that PKC activity is not required for short-term LFI. Inhibitors alone did not significantly affect responses 90 min after injection (Total Response Time [min]: controls 29.6 ± 3.2 [$n = 17$], chelerythrine 40.0 ± 7.5 [$n = 6$], Bis 25.5 ± 2.6 [$n = 6$], ANOVA $P = 0.17$; Time in the Mouth [min]: controls 26.0 ± 3.6 ; chelerythrine 38.1 ± 7.9 ; Bis 22.3 ± 3.2 , ANOVA $P = 0.16$). Although it may seem surprising that neither PKA nor PKC are required for short-term LFI memory as these kinases appear prominent in *Aplysia* in vitro paradigms, our experiments do not preclude the activation of these kinases with training or even a modulatory role of these kinases in STM. As the same LFI training paradigm induces STM and LTM, our experiments demonstrate that while training may broadly induce the activation of many kinase and signaling pathways, only the activation of a subset of pathways is critical for STM in vivo.

Memory depends not only upon factors involved in induction and consolidation, but also those components necessary for maintenance and recall. To more fully understand the role of PKA and PKC in LFI memory, we tested whether these pathways were necessary for the expression or retrieval of memory. To evaluate STM, animals were trained and injected immediately after training with either H89 to inhibit PKA or chelerythrine to inhibit PKC. Animals were then tested for STM 30 min later. Neither PKA nor PKC appeared necessary for the maintenance or recall of short-term LFI as treated animals displayed STM comparable to controls (Fig. 3A; Supplemental Fig. 5). We performed similar experiments to test the recall of LTM. Animals were trained and then injected with H89, chelerythrine, or vehicle 30 min prior to testing (Fig. 3B). All groups exhibited significant LTM suggesting that neither PKA nor PKC is involved in the maintenance of memory at this late stage or necessary for recall of the memory.

During LFI training, nitric oxide and histamine signal failed swallowing attempts (Katzoff et al. 2002, 2006, 2010). For short-term plasticity, NO-cGMP signaling may potentially modulate cyclic nucleotide gated calcium channels. During LTM formation, the recruitment of additional kinase pathways occurs followed by the requirement for polyADP-ribosylation (Cohen-Armon et al. 2004) and induction of the transcription factor CCAAT/enhancer binding protein (ApC/EBP) (Levitan et al. 2008). NO-cGMP signaling could activate the cAMP-PKA pathway for long-term LFI as proposed for LTM in honeybees and crickets (Müller 2000; Matsumoto et al. 2006, 2009). Furthermore, we hypothesize that PKC functions early during LTM with subsequent persistent PKA activation, CREB-dependent transcription and induction of *ApC/EBP*. Thus, the identification of mechanistic differences in the requirement for PKA and PKC between short- and long-term negatively reinforced operant memory highlights the complexity of signaling cascades underlying memory in a relatively simple model system.

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