

Cell-Specific PKM Isoforms Contribute to the Maintenance of Different Forms of Persistent Long-Term Synaptic Plasticity

Jiangyuan Hu,¹ Kerry Adler,¹ Carole Abi Farah,² Margaret H. Hastings,³ Wayne S. Sossin,^{2,3} and Samuel Schacher¹

¹Department of Neuroscience, Columbia University Medical Center, New York State Psychiatric Institute, New York, New York 10032, ²Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec H3A 2B4, Canada, and ³Department of Psychology, McGill University, Montreal Neurological Institute, Montreal, Quebec H3A 1B1, Canada

Multiple kinase activations contribute to long-term synaptic plasticity, a cellular mechanism mediating long-term memory. The sensorimotor synapse of *Aplysia* expresses different forms of long-term facilitation (LTF)—nonassociative and associative LTF—that require the timely activation of kinases, including protein kinase C (PKC). It is not known which PKC isoforms in the sensory neuron or motor neuron L7 are required to sustain each form of LTF. We show that different PKMs, the constitutively active isoforms of PKCs generated by calpain cleavage, in the sensory neuron and L7 are required to maintain each form of LTF. Different PKMs or calpain isoforms were blocked by overexpressing specific dominant-negative constructs in either presynaptic or postsynaptic neurons. Blocking either PKM Apl I in L7, or PKM Apl II or PKM Apl III in the sensory neuron 2 d after 5-hydroxytryptamine (5-HT) treatment reversed persistent nonassociative LTF. In contrast, blocking either PKM Apl II or PKM Apl III in L7, or PKM Apl II in the sensory neuron 2 d after paired stimuli reversed persistent associative LTF. Blocking either classical calpain or atypical small optic lobe (SOL) calpain 2 d after 5-HT treatment or paired stimuli did not disrupt the maintenance of persistent LTF. Soon after 5-HT treatment or paired stimuli, however, blocking classical calpain inhibited the expression of persistent associative LTF, while blocking SOL calpain inhibited the expression of persistent nonassociative LTF. Our data suggest that different stimuli activate different calpains that generate specific sets of PKMs in each neuron whose constitutive activities sustain long-term synaptic plasticity.

Key words: *Aplysia*; calpain; cell culture; long-term facilitation; PKC; PKM

Significance Statement

Persistent synaptic plasticity contributes to the maintenance of long-term memory. Although various kinases such as protein kinase C (PKC) contribute to the expression of long-term plasticity, little is known about how constitutive activation of specific kinase isoforms sustains long-term plasticity. This study provides evidence that the cell-specific activities of different PKM isoforms generated from PKCs by calpain-mediated cleavage maintain two forms of persistent synaptic plasticity, which are the cellular analogs of two forms of long-term memory. Moreover, we found that the activation of specific calpains depends on the features of the stimuli evoking the different forms of synaptic plasticity. Given the recent controversy over the role of PKM ζ maintaining memory, these findings are significant in identifying roles of multiple PKMs in the retention of memory.

Introduction

Persistent changes in synaptic strength contribute to long-term memories (Kandel, 2012; Maren, 2005; Caroni et al., 2012; Col-

lingridge et al., 2013; Takeuchi et al., 2014; Bailey et al., 2015; Basu and Siegelbaum, 2015). Such changes are mediated by the modulation of gene expression (Abraham et al., 1991; Alberini et al., 1994; Impey et al., 1996; Lanahan and Worley, 1998; Alberini and Kandel, 2014; Veyrac et al., 2014), protein synthesis (Martin et al., 1997a; Sutton and Schuman, 2006; Abraham and Williams, 2008; Costa-Mattioli et al., 2009; Redondo and Morris, 2011; Santini et al., 2014), and kinase activations (Malinow et al., 1988; Lisman, 1994; Thiels and Klann, 2001; Mayford, 2007). Different kinase

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Correspondence should be addressed to Jiangyuan Hu, Department of Neuroscience, Columbia University Medical Center, New York State Psychiatric Institute, 1051 Riverside Drive, New York, NY 10032. E-mail: jh2004@cumc.columbia.edu.

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isoforms contribute to the expression of long-term plasticity in the mammalian CNS (Klann et al., 1991; Thomas et al., 1994; Abel et al., 1997; Schafe et al., 1999; Huang et al., 2000; Duffy and Nguyen, 2003; Yang et al., 2004; Nie et al., 2007; Kandel, 2012; Tsokas et al., 2016) and at the sensorimotor synapse of *Aplysia* (Hegde et al., 1993; Sossin et al., 1994; Martin et al., 1997b; Chain et al., 1999; Purcell et al., 2003; Liu et al., 2004; Fiumara et al., 2015). Despite this wealth of data, little is known about whether coordinated and constitutive activations of specific kinase isoforms in the presynaptic and postsynaptic compartments are needed to sustain long-term plasticity.

The sensorimotor synapse of *Aplysia* expresses different forms of persistent long-term facilitation (LTF). Nonassociative LTF is produced by repeated applications of serotonin [5-hydroxytryptamine (5-HT)] or repeated sensitizing stimuli and is a cellular analog of long-term sensitization (Castellucci et al., 1978; Frost et al., 1985; Montarolo et al., 1986; Cleary et al., 1998; Marinesco et al., 2006; Hu et al., 2011; Hu and Schacher, 2014). Associative LTF is produced by pairing activity in the sensory neuron with 5-HT or pairing tactile stimulation with sensitizing stimuli, and is a cellular analog of long-term classical conditioning (Carew et al., 1981; Walters and Byrne, 1983; Buonomano and Byrne, 1990; Hu and Schacher, 2015). Each form of LTF requires timely activation of protein kinase A (PKA), MAPK, and PKC (Abrams et al., 1991; Martin et al., 1997a; Chain et al., 1999; Hu et al., 2004, 2006, 2007, 2011; Liu et al., 2004; Shobe et al., 2009; Villareal et al., 2009; Fiumara et al., 2015). Persistent LTF (lasting more than a week) for each form is evoked by two sets of stimuli in 2 consecutive days or after a 5 h interval, and incubation with the PKC inhibitor chelerythrine resulted in the reversal of nonassociative or associative LTF (Cai et al., 2011; Hu et al., 2011; Hu and Schacher, 2015). *Aplysia* neurons express the following three isoforms of PKC (Kruger et al., 1991; Bougie et al., 2009): PKC Apl I (classical); PKC Apl II (novel); and PKC Apl III (atypical). Each of these PKC isoforms can be converted to a constitutively active form after calpain-dependent cleavage, PKM Apl I, PKM Apl II, and PKM Apl III, some of which contribute to intermediate forms of facilitation (Sossin, 2007; Sutton et al., 2004; Bougie et al., 2009, 2012; Farah et al., 2016). Which isoforms of PKM in each neuron need to be active to maintain the two forms of persistent LTF?

We manipulated the activity of PKC/PKM isoforms or two calpain isoforms, classical and atypical small optic lobe (SOL), using a dominant-negative (dn) approach. We found that cell-specific activations of different PKMs were required to sustain different forms of persistent synaptic plasticity. Calpain activity was not required to maintain persistent LTF, but specific calpain isoform activity was required for the expression of each form of persistent LTF. Thus, different stimuli activate different calpain isoforms that generate specific PKMs in the sensory neurons and L7, whose constitutive activities maintain different forms of long-term plasticity.

Materials and Methods

Cell culture and electrophysiology. Sensory neurons were isolated from pleural ganglia dissected from adult animals (60–80 g; *Aplysia californica*, which are hermaphrodites; RRID: SCR_008361), and motor neuron L7s were isolated from juvenile abdominal ganglia (2 g) and maintained in coculture up to 10 d (Hu et al., 2015). The coculture contained one sensory neuron and one L7. Each culture represented one sample, since each motor neuron L7 was derived from a separate animal. The sensory neurons added to each L7 for cultures prepared on the same day (between 9 and 13 cultures) were derived from the pleural ganglia of two adult animals (four hemi-ganglia in total). The cultures prepared on

the same day were divided into two experimental and two control (Cont) groups for each experiment. Cultures were maintained at 18°C and fed every other day with medium containing 50% filtered hemolymph and 50% L15.

A standard intracellular recording technique was used to record the amplitude of EPSPs evoked in motor neuron L7. The soma of L7 was impaled with a recording microelectrode (10–15 M Ω) filled with a solution containing 2.0 M K-acetate, 0.5 M KCl, and 10 mM K-HEPES, pH 7.4. An AxoClamp-2A amplifier and pCLAMP software (Molecular Devices) were used for data acquisition. To accurately record the amplitude of the EPSPs in each coculture, the membrane potential of L7 was hyperpolarized to -80 mV (~ 25 mV below resting potential), and an EPSP was evoked in L7 by stimulating the sensory neuron with a brief (0.3–0.5 ms) depolarizing pulse to evoke an action potential using an extracellular electrode placed near the cell body of the sensory neuron. EPSP amplitudes were monitored before and after various treatments at the indicated time points in the different experiments. The resting potential or input resistance measured in the soma of L7 was not significantly altered by time in culture, stimulation, and/or overexpression of control or experimental constructs. These measures of cell viability changed by $<10\%$ over the course of the experiments (6 d, days 4–10 in culture). Cultures on day 4 with initial EPSP amplitudes in the range of 15–30 mV were selected (85% of total cultures) for control and experimental conditions. The synaptic strength on day 4 in culture represented the basal synaptic strength (defined as the synaptic baseline), since there was little change in synapse strength over the next week in the absence of stimulation (Hu et al., 2011; Hu and Schacher, 2014, 2015; see Results). The assignment of any culture to control and experimental groups at the start of each experiment on day 4 in culture (defined as day 0 in Results and figures) was to ensure that there were no significant differences in the mean and variance of the initial strength of the synapses between the groups before treatments.

Persistent LTF. Persistent nonassociative LTF lasting more than a week was evoked as reported previously (Hu et al., 2011). After recording the initial EPSP amplitudes of sensorimotor synapses on day 0, some cultures were exposed to 2 consecutive days (days 0 and 1) of 5 applications of 5-HT (5 μ M; Sigma-Aldrich) each lasting 5 min at 20 min intervals (2 5-HT; see Fig. 1A) to evoke persistent nonassociative LTF. Each application of 5-HT was washed out with a solution containing 50% seawater and 50% L15. Control cultures received mock treatments. EPSP amplitudes were re-examined on day 3 (2 d after the last set of applications) before the injections of constructs and at subsequent time points, as indicated in the protocol portion of the figures.

Persistent associative LTF lasting more than a week was evoked by two pairings of a presynaptic tetanus with an application of 5-HT on 2 consecutive days [days 0 and 1; 2 (Tet plus 5-HT); see Fig. 7A], as reported previously (Hu and Schacher, 2015). In brief, after recording the initial EPSP amplitudes on day 0, the sensory neuron was stimulated with a tetanus (20 Hz for 2 s) that was followed immediately (0.5–1.5 s) by an application of 5-HT (5 μ M) for 5 min. During the tetanus, motor neuron L7 was maintained at its resting membrane potential (-55 to -60 mV). Cultures were rinsed with L15-sea water and then with culture medium (50% filtered hemolymph and 50% L15). On day 1, these cultures received a second presynaptic tetanus paired with an application of 5-HT. Control cultures received mock treatments, and in some cultures cells received unpaired stimulation where the application of tetanus and 5-HT were separated by 3–5 min (unpaired). Two unpaired stimuli evoked no LTF (Hu and Schacher, 2015). The strengths of the synapses were re-examined on day 3 (2 d after the last pairing) and at subsequent time points, as indicated in the protocol portion of the figures.

Plasmid constructs and microinjection. All DNA constructs use the pNEX3 plasmid background and promoter (Kaang, 1996). Dominant-negative monomeric red fluorescent protein (mRFP)-PKMs and dominant-negative mRFP-PKC Apl III have a conserved aspartic acid in the catalytic site converted to alanine (Apl III D392-A, Apl I D444-A, and Apl II D526-A). This mutation removes $>95\%$ of kinase activity but allows for stabilizing phosphorylation at critical priming sites (Cameron et al., 2009; Bougie et al., 2012; Farah et al., 2016). The dominant-negative mRFP-PKM Apl III and mRFP-PKM Apl I constructs have been shown

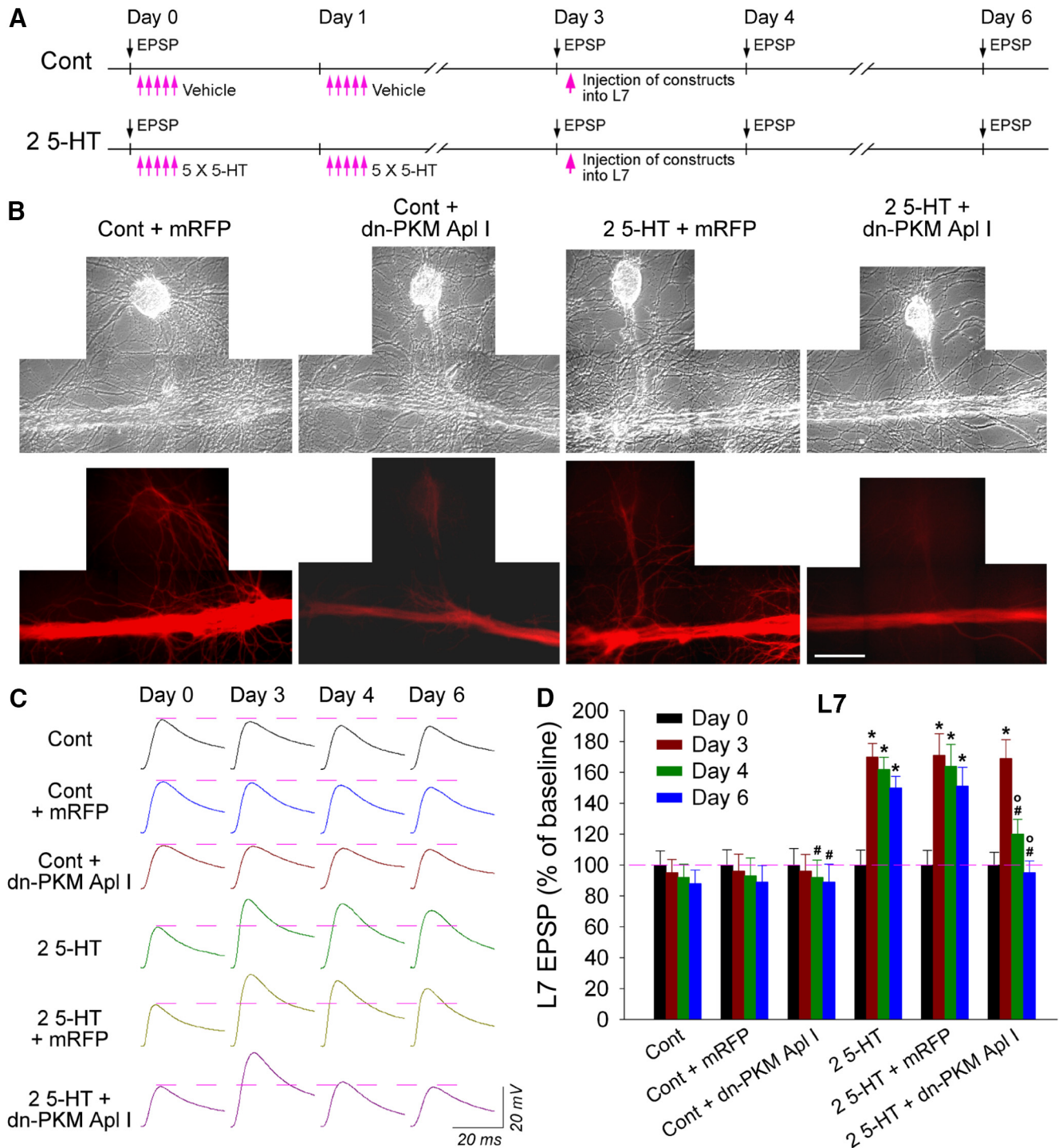


Figure 1. PKM Apl I activity in L7 is required after day 3 to maintain persistent nonassociative LTF. **A**, Experimental protocol for evoking persistent nonassociative LTF consists of repeated applications of 5-HT on days 0 and 1. EPSP amplitudes were tested before control or experimental treatments on day 0 and retested on days 3, 4, and 6. Various constructs (control or experimental) were injected into L7 on day 3. **B**, Phase contrast views of sensorimotor cultures and epifluorescent views of construct expression in the same view area 24 h after injection. Scale bar, 50 μ m. **C**, EPSPs in L7 over time for control and experimental groups. Repeated applications of 5-HT resulted in an increase in EPSP amplitude that persisted to day 6, except for the experimental group where L7 overexpressed dn-PKM Apl I (2 5-HT + dn-PKM Apl I). Overexpression of dn-PKM Apl I did not affect the synaptic baseline in the control group. **D**, Summary of the changes in EPSP amplitudes for control and experimental groups. A two-factor ANOVA indicated a significant effect of treatment over time ($F_{(15,135)} = 40.72; p < 0.001$). Individual comparisons indicated the following: (1) overexpression of dn-PKM Apl I in L7 after control treatment (Cont + dn-PKM Apl I) had no significant effect on the synaptic baseline compared with control or overexpressing mRFP in control (#) on day 4 ($F_{(5,45)} = 0.002$ or 0.001 ; all $p > 0.8$) and day 6 ($F_{(5,45)} = 0.0003$ or 0.0002 ; all $p > 0.9$); (2) two sets of 5-HT treatment (2 5-HT) produced a significant increase in synaptic strength (*) on days 3, 4, and 6 compared with Cont, Cont + mRFP, or Cont + dn-PKM Apl I ($F_{(5,45)} = 3.859, 3.962, \text{ or } 4.649$, all $p < 0.01$ on day 3; $F_{(5,45)} = 3.981, 3.938, \text{ or } 3.457$, all $p < 0.01$ on day 4; $F_{(5,45)} = 3.923, 3.829, \text{ or } 3.237$, all $p < 0.01$ on day 6); (3) overexpressing control construct mRFP (2 5-HT + mRFP) on day 3 did not significantly affect the increase in synaptic strength produced by two sets of 5-HT treatments on days 4 and 6 compared with 2 5-HT alone ($F_{(5,45)} = 0.003$ and 0.002 ; all $p > 0.9$); (4) overexpressing dn-PKM Apl I on day 3 resulted in a significant reversal of the increase in synaptic strength by 2 5-HT (2 5-HT + dn-PKM Apl I) on days 4 and 6 (o) compared with other 5-HT-treated groups ($F_{(5,45)} = 2.772$ and 3.769 , all $p < 0.05$ compared with 2 5-HT alone; $F_{(5,45)} = 2.946$ and 3.944 , all $p < 0.05$ compared with 2 5-HT + mRFP); and (5) the synaptic strength in 2 5-HT + dn-PKM Apl I was not significantly different from each control group (#) on days 4 and 6 ($F_{(5,45)} = 0.79$ and 0.069 , all $p > 0.5$ compared with Cont; $F_{(5,45)} = 0.769$ and 0.056 , all $p > 0.5$ compared with Cont + mRFP; $F_{(5,45)} = 0.677$ and 0.04 , all $p > 0.5$ compared with Cont + dn-PKM Apl I).

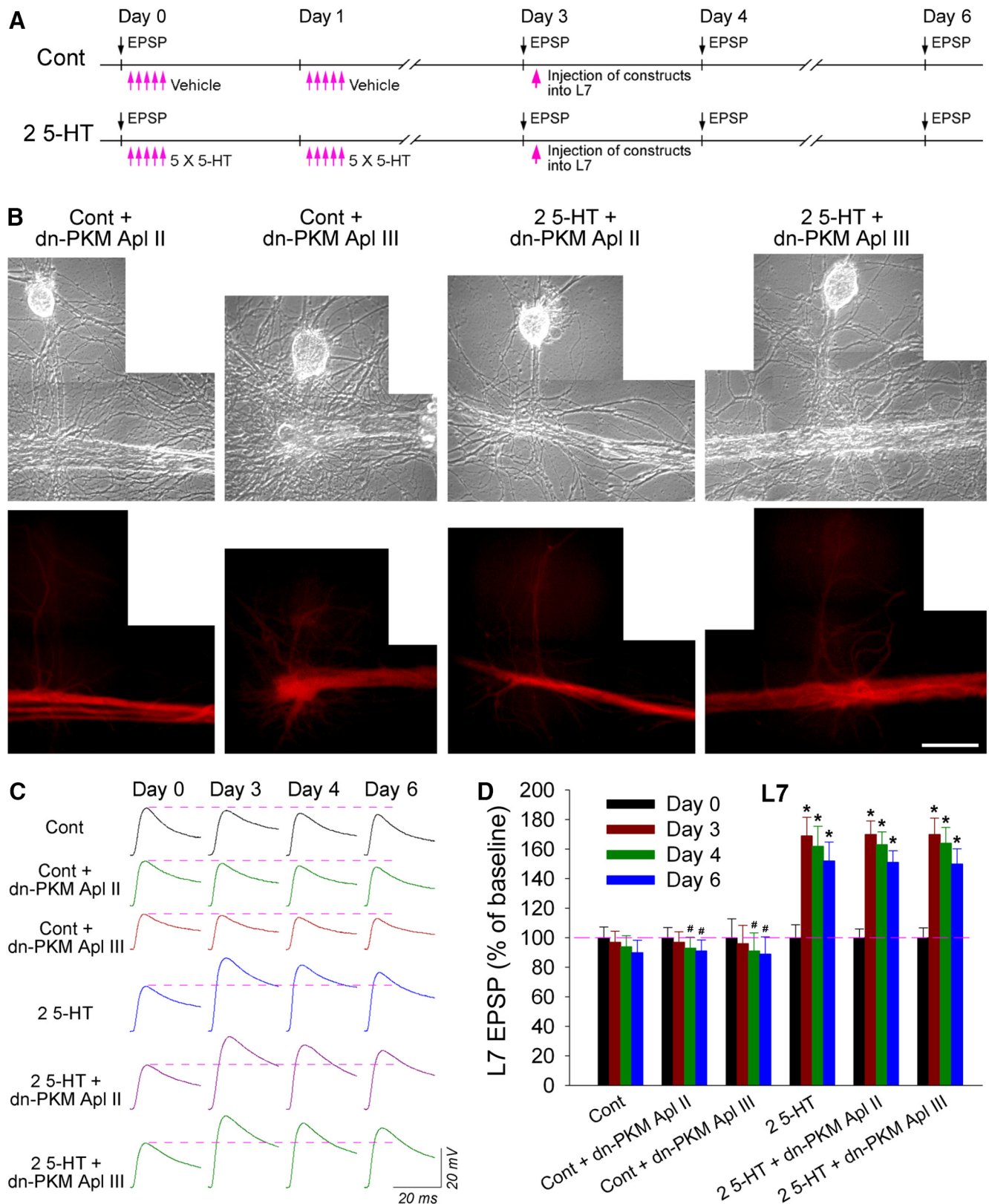


Figure 2. PKM Apl II or PKM Apl III activity in L7 is not required after day 3 to maintain nonassociative LTF. **A**, Experimental protocol. Persistent nonassociative LTF was evoked by repeated applications of 5-HT on days 0 and 1. Various constructs were injected into L7 on day 3. **B**, Phase contrast views of sensorimotor cultures and epifluorescent views of construct expression in the same view area 24 h after injection. Scale bar, 50 μ m. **C**, Examples of the change in the EPSP amplitudes in L7 over time for control and experimental groups. All experimental groups, receiving repeated applications of 5-HT (2 5-HT), showed an increase in EPSP amplitude that persisted to day 6, including those that were overexpressing dn-PKM Apl II or dn-PKM Apl III in L7. Overexpression of the dominant-negative constructs did not affect synaptic baseline in control. **D**, Summary of the change in EPSP amplitudes for control and experimental groups. A two-factor ANOVA indicated a significant effect of treatment over time ($F_{(15,153)} = 42.479; p < 0.001$). Individual comparisons indicated the following: (1) overexpression of dn-PKM Apl II or dn-PKM Apl III on day 3 in L7 after control treatment (Cont + dn-PKM Apl II; Cont + dn-PKM Apl III) had no significant effect on synaptic baseline compared with control (#) on days 4 and 6 (Figure legend continues.)

to block distinct forms of intermediate forms of facilitation (Bougie et al., 2012; Farah et al., 2016). Dominant-negative forms of enhanced green fluorescent protein (eGFP)-PKC Apl I and II were made previously (Manseau et al., 2001). Dominant-negative eGFP-PKC Apl I and eGFP-PKC Apl II have a catalytic lysine converted to arginine, and the priming phosphorylation sites mutated were to glutamic acid for stabilization. These dominant-negatives have been shown to be effective at having isoform-specific effects at inhibiting physiological changes that are produced by activating their respective PKC isoforms (Manseau et al., 2001; Zhao et al., 2006; Lorenzetti et al., 2008; Hu et al., 2010). While PKCs do not dimerize, dominant-negative PKMs and PKCs compete for scaffolds and substrates with the endogenous PKCs, thus blocking their functional activity. The classical and SOL calpains were the most prominent calpains expressed in sensory and motor neurons, although other calpains also exist in *Aplysia* databases (Farah et al., 2016). The calpains were cloned by PCR based on transcriptome data (Farah et al., 2016), and dominant-negative calpains were generated by converting the catalytic cysteine to serine (Farah et al., 2016). Many calpains act as dimers, and dominant-negative calpains could act by forming mutant heterodimers. Alternatively, dominant-negative calpains, similar to dominant-negative PKCs, may act by competing with endogenous calpains for substrates and scaffolds. The dominant-negative classical calpain (dn-classical) has been shown to be effective at blocking both the cleavage of PKCs using a FRET-based live imaging assay and the induction of intermediate facilitation, while dominant-negative SOL calpain (dn-SOL) did not have a significant effect in these experiments (Farah et al., 2016).

Microinjection was executed by a pneumatic picopump (PV820, World Precision Instruments). A solution of constructs (0.3 $\mu\text{g}/\mu\text{l}$ DNA in distilled water) containing 0.2% Fast Green was microinjected into the nuclei of sensory neurons or L7s on day 1 or 3 (see the protocol for each experiment). The tip of the micropipette was inserted into the cell nucleus. A short pressure pulse (20–50 ms duration; 20–40 psi) was delivered until the nucleus of the sensory neurons became uniformly green (Hu et al., 2010). Because the volume of the nucleus in L7 is $\sim 30\times$ that of the nucleus of the sensory neuron, the pressure pulse (20–40 psi) of 600–1200 ms was delivered until the nucleus of L7 became uniformly green. The fluorescent images of construct expression were captured with a Nikon Diaphot microscope 24 h after injections.

Quantification and data analysis. All data are expressed as the mean \pm SEM produced by the indicated treatments. The EPSP amplitude was measured in millivolts (mV). The initial EPSP amplitudes on day 0 were normalized as 100%. The changes in EPSP amplitudes were measured by dividing the EPSP amplitudes on day 2, 3, 4, 5, or 6 by the EPSP amplitude on day 0 multiplied by 100%. Some cultures expressing persistent LTF (8%) had an increase in synaptic strength that was expressed as an action potential evoked in L7 on day 2 or 3 with the test stimulus to the sensory neurons. These cultures were excluded from all analyses. Overall, 91% of the remaining cultures survived to the final test point (day 5 or 6). All images were viewed with a Nikon Diaphot microscope attached to a silicon-intensified target video camera (Dage Series 68, Dage-MTI). Fluorescent intensity (arbitrary units) was measured by the Microcomputer-Controlled Imaging Device

←

(Figure legend continued.) ($F_{(5,51)} = 0.0001$ and 0.002 , all $p > 0.8$ for dn-PKM Apl II; $F_{(5,51)} = 0.003$ and 0.0001 , all $p > 0.7$ for dn-PKM Apl III); (2) 2 5-HT produced a significant increase in synaptic strength compared with each control group (*) on days 3, 4, and 6 ($F_{(5,51)} = 2.997$, 2.758 , and 2.525 , all $p < 0.05$ compared with Cont; $F_{(5,51)} = 3.423$, 3.222 , and 2.744 , all $p < 0.05$ compared with Cont + dn-PKM Apl II; $F_{(5,51)} = 3.58$, 3.4 , and 2.929 , all $p < 0.01$ compared with Cont + dn-PKM Apl III); (3) overexpression of dn-PKM Apl II on day 3, the changes in synaptic strength evoked by 2 5-HT (2 5-HT + dn-PKM Apl II) remained significantly greater than those in each control group (*) on days 4 and 6 ($F_{(5,51)} = 3.593$ and 3.174 , all $p < 0.01$ compared with Cont; $F_{(5,51)} = 4.381$ and 3.6 , all $p < 0.01$ compared with Cont + dn-PKM Apl II) but were not significantly different from 2 5-HT alone ($F_{(5,51)} = 0.001$ and 0.0001 , all $p > 0.9$); (4) overexpressing dn-PKM Apl III on d 3, the change in synaptic strength evoked by 2 5-HT (2 5-HT + dn-PKM Apl III) remained significantly greater than that for each control group (*) on days 4 and 6 ($F_{(5,51)} = 3.86$ and 3.16 , all $p < 0.01$ compared with Cont; $F_{(5,51)} = 5.004$ and 3.863 , all $p < 0.01$ compared with Cont + dn-PKM Apl III) but were not significantly different from 2 5-HT alone ($F_{(5,51)} = 0.003$ and 0.002 ; all $p > 0.8$).

software package (Imaging Research). ANOVA was used to assess overall significant differences, and Scheffé's F test was used to gauge significant differences between individual treatments. Significant differences between specific groups in the histograms summarizing the changes in synaptic strength were indicated as follows: treatments that evoked a significant change ($p < 0.05$) compared with controls are designated with an asterisk (*), treatments that resulted in changes in synaptic strength and were not significantly different from controls at any time point are designated with a hashtag (#), treatments that resulted in significant changes in synaptic strength after stimuli such that at specific time points they were significantly different from other stimulated groups are designated with a small circle (○).

Results

The role of PKC/PKM activities in maintaining long-term synaptic plasticity in mammals is controversial (Volk et al., 2013; Jalil et al., 2015; Tsokas et al., 2016)—compensatory substitutions are recruited under conditions when some isoforms are knocked down or out, suggesting roles for multiple PKC isoforms in long-term plasticity. In *Aplysia*, constitutive PKC activity is required for sustaining long-term plasticity at the behaviorally relevant sensorimotor synapse (Cai et al., 2011; Hu et al., 2011; Hu and Schacher, 2015). It is unclear, however, which isoforms of PKC or the constitutively active PKMs in the presynaptic and postsynaptic partner contributes to the persistence. We therefore used a dominant-negative approach to interfere with the activity of specific PKM or PKC isoforms on day 3 in either L7 or sensory neurons to determine whether the activities of specific isoforms starting 2 d after repeated applications of 5-HT or paired stimulation contributed to different forms of persistent plasticity. All of the dominant-negative constructs were kinase dead ($>95\%$) but were otherwise homologous to wild type to allow interactions with endogenous partners. As controls, we also examined whether the synaptic baseline was affected by reducing the activity of specific PKM or PKC isoforms in either L7 or sensory neurons starting on day 3.

Different cell-specific PKM isoforms maintain persistent nonassociative LTF

Because constitutive PKC activity contributes to the maintenance of LTF (Cai et al., 2011; Hu and Schacher, 2015), we focused on the potential roles of the *Aplysia* PKM isoforms in L7 or sensory neurons by interfering with specific PKM isoform activity (Cameron et al., 2009; Bougie et al., 2012; Farah et al., 2016) starting 2 d after the last 5-HT application (Fig. 1A). The overexpression of the dominant-negative mRFP-tagged PKM isoforms and control construct (mRFP) were monitored by fluorescent microscopy 24 h after the construct was injected on day 3 (Figs. 1B, 2B; see also 4B, 5B). The mRFP control construct was expressed quite strongly and was detected throughout the sensory neuron or L7, while the mRFP-tagged dominant-negative constructs for each of the PKMs was expressed at equivalent levels in the cell body, axons, and neurites of L7 or sensory neurons independent of any changes in synaptic strength produced by a previous stimulation history on day 0 or 1 (Figs. 1B, 2B; see also 4B, 5B; data not shown). We examined the consequences of overexpressing each control or dominant-negative construct in L7 or sensory neurons on the persistence of nonassociative LTF produced by 2 d of brief applications of 5-HT (2 5-HT).

Overexpression of dn-mRFP-PKM Apl I in L7 resulted in the reversal of persistent nonassociative LTF (2 5-HT plus dn-PKM Apl I; $n = 13$; Fig. 1C,D). The decline in synaptic strength on days 4 and 6 was significant compared with the persistent nonassociative LTF expressed in cultures with no construct injection (2 5-HT; $n = 8$) or after injection of the control construct (2 5-HT

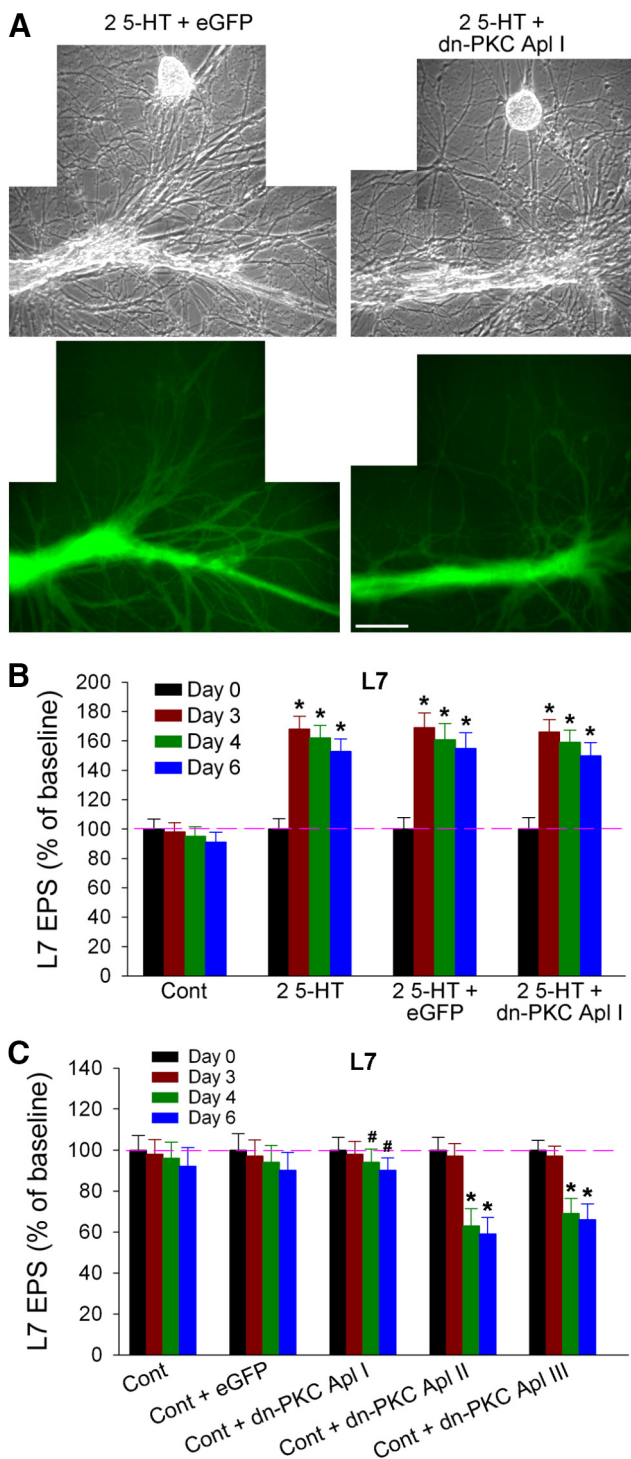


Figure 3. PKM Apl I activity in L7 is not required after day 3 to maintain nonassociative LTF. **A**, Phase contrast views of sensorimotor cultures and epifluorescent views of construct expression in the same view area (control and dominant-negative forms) in L7 24 h after injection. Scale bar, 50 μ m. **B**, Summary of the change in the EPSP amplitude for control and experimental groups indicated that overexpression of dn-PKC Apl I in L7 (2 5-HT + dn-PKC Apl I) failed to affect persistent nonassociative LTF. A two-factor ANOVA indicated a significant effect of treatment over time ($F_{(9,87)} = 55.135$; $p < 0.001$). Individual comparisons indicated the following: (1) stimulation with 5-HT produced a significant change in synaptic strength compared with control (*) on days 3, 4, and 6 ($F_{(3,29)} = 9.603, 8.779, \text{ and } 7.166$; all $p < 0.01$); (2) overexpressing control construct eGFP on day 3 had no significant effect on the change in synaptic strength evoked by 2 d of 5-HT treatments (2 5-HT + eGFP), which remained significantly greater than the change observed in control (*) on days 4 and 6 ($F_{(3,29)} = 8.84 \text{ and } 7.12$; all $p < 0.01$); and (3) After 2 d of 5-HT treatments, overexpression of dn-PKC Apl I (2 5-HT + dn-PKC Apl I) did not

significantly affect the change in synaptic strength compared with other 5-HT-treated groups on days 4 and 6 ($F_{(3,29)} = 0.021 \text{ and } 0.022$, all $p > 0.8$ compared with 2 5-HT alone; $F_{(3,29)} = 0.007 \text{ and } 0.051$, all $p > 0.8$ compared with 2 5-HT + eGFP), which remained significantly greater than the change in control ($F_{(3,29)} = 8.84 \text{ and } 7.12$, all $p < 0.01$). **C**, Summary of the change in the EPSP amplitudes for various control groups with and without overexpression of the control construct or the dominant-negative constructs. A two-factor ANOVA indicated a significant effect of treatment over time ($F_{(12,123)} = 23.302$; $p < 0.001$). Individual comparisons indicated the following: (1) overexpression of dn-PKC Apl I in L7 on day 3 after control treatment (Cont + dn-PKC Apl I) had no significant effect on synaptic baseline compared with control or control + eGFP (#) on day 4 ($F_{(4,41)} = 0.008 \text{ or } 0.003$; all $p > 0.8$) and day 6 ($F_{(4,41)} = 0.007 \text{ or } 0.004$; all $p > 0.8$); (2) overexpression of dn-PKC Apl II in L7 on day 3 after control treatment (Cont + dn-PKC Apl II) had a significant effect (depression) on synaptic baseline compared with control or control + eGFP (*) on day 4 ($F_{(4,41)} = 2.203 \text{ or } 2.097$; all $p < 0.05$) and day 6 ($F_{(4,41)} = 1.997 \text{ or } 2.113$; all $p < 0.05$); (3) overexpression of dn-PKC Apl III in L7 after control treatment on day 3 (Cont + dn-PKC Apl III) had a significant effect (depression) on synaptic baseline compared with control or control + eGFP (*) on day 4 ($F_{(4,41)} = 2.211 \text{ or } 1.989$; all $p < 0.05$) and day 6 ($F_{(4,41)} = 1.933 \text{ or } 1.987$; all $p < 0.05$).

plus mRFP; $n = 8$). The persistent facilitation was maintained on days 4 and 6 in these latter two groups compared with Cont ($n = 6$) or with overexpression of the control construct (Cont plus mRFP; $n = 8$). The decline in synaptic strength produced by the overexpression of dn-PKM Apl I was such that it was no longer significantly different from the control groups. Overexpression of dn-PKM Apl I in L7 after control treatment (Cont plus dn-PKM Apl I; $n = 6$) did not alter the synaptic baseline compared with control alone (Cont; $n = 6$; Fig. 1C,D). Thus, PKM Apl I activity in L7 contributes to the maintenance of persistent nonassociative LTF.

Persistent nonassociative LTF was not reversed when overexpressing either dn-PKM Apl II (2 5-HT plus dn-PKM Apl II; $n = 13$) or dn-PKM Apl III (2 5-HT plus dn-PKM Apl III; $n = 15$) in L7 starting 2 d after 5-HT treatments (Fig. 2). The increases in synaptic strength in both sets of cultures were comparable to the increase evoked by 2 5-HT ($n = 7$). In the absence of any 5-HT applications, the synaptic baseline was unaffected by the overexpression of dn-PKM Apl II (Cont plus dn-PKM Apl II; $n = 8$) or by dn-PKM Apl III (Cont plus dn-PKM Apl III; $n = 8$) compared with Cont ($n = 6$; Fig. 2C,D). Thus, reducing either PKM Apl II or PKM Apl III activity in L7 does not affect the maintenance of persistent nonassociative LTF.

We also examined whether the activity of classical PKC (PKC Apl I), novel PKC (PKC Apl II), or atypical PKC (PKC Apl III) in L7 or the sensory neurons might contribute to the maintenance of persistent nonassociative LTF. While dominant-negative PKCs and PKMs might be expected to have similar effects, the dominant-negative PKCs contain regulatory domain elements absent in the PKMs that may allow them to interact with additional scaffolds or substrates distinct from the PKMs. Alternatively, while all domains in PKM are present in PKC, inhibitory interactions between the regulatory domain and the catalytic domain may mask these interactions in dominant-negative PKCs, but not PKMs, and allow dominant-negative PKMs to have activities not shared with dominant-negative PKCs. The overexpression of the dominant-negative eGFP-tagged PKC isoforms and control construct (eGFP) were monitored by fluorescent microscopy 24 h after constructs were injected on day 3 (Fig. 3A; also see 6A). The eGFP construct was expressed quite strongly and was detected throughout L7 or sensory neurons, while each of the eGFP-tagged dominant-negative PKC constructs was expressed at equivalent levels in the cell body, axons, and neurites of L7 or sensory neurons independent of the previous stimulation history (data not shown). Each dominant-negative PKC con-

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significantly affect the change in synaptic strength compared with other 5-HT-treated groups on days 4 and 6 ($F_{(3,29)} = 0.021 \text{ and } 0.022$, all $p > 0.8$ compared with 2 5-HT alone; $F_{(3,29)} = 0.007 \text{ and } 0.051$, all $p > 0.8$ compared with 2 5-HT + eGFP), which remained significantly greater than the change in control ($F_{(3,29)} = 8.84 \text{ and } 7.12$, all $p < 0.01$). **C**, Summary of the change in the EPSP amplitudes for various control groups with and without overexpression of the control construct or the dominant-negative constructs. A two-factor ANOVA indicated a significant effect of treatment over time ($F_{(12,123)} = 23.302$; $p < 0.001$). Individual comparisons indicated the following: (1) overexpression of dn-PKC Apl I in L7 on day 3 after control treatment (Cont + dn-PKC Apl I) had no significant effect on synaptic baseline compared with control or control + eGFP (#) on day 4 ($F_{(4,41)} = 0.008 \text{ or } 0.003$; all $p > 0.8$) and day 6 ($F_{(4,41)} = 0.007 \text{ or } 0.004$; all $p > 0.8$); (2) overexpression of dn-PKC Apl II in L7 on day 3 after control treatment (Cont + dn-PKC Apl II) had a significant effect (depression) on synaptic baseline compared with control or control + eGFP (*) on day 4 ($F_{(4,41)} = 2.203 \text{ or } 2.097$; all $p < 0.05$) and day 6 ($F_{(4,41)} = 1.997 \text{ or } 2.113$; all $p < 0.05$); (3) overexpression of dn-PKC Apl III in L7 after control treatment on day 3 (Cont + dn-PKC Apl III) had a significant effect (depression) on synaptic baseline compared with control or control + eGFP (*) on day 4 ($F_{(4,41)} = 2.211 \text{ or } 1.989$; all $p < 0.05$) and day 6 ($F_{(4,41)} = 1.933 \text{ or } 1.987$; all $p < 0.05$).

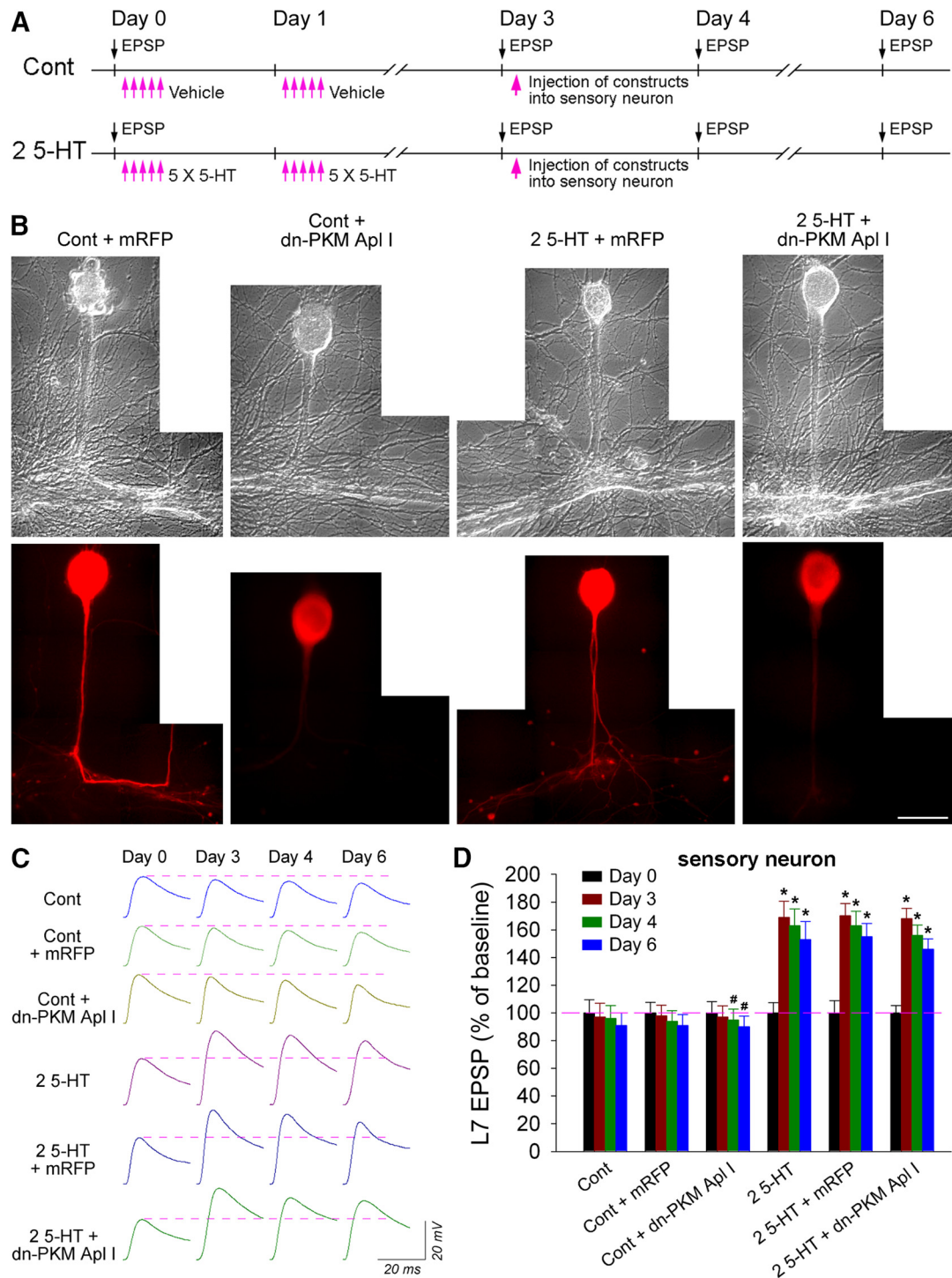


Figure 4. PKM Apl I activity in the sensory neurons is not required after day 3 to maintain nonassociative LTF. **A**, Experimental protocol for evoking persistent nonassociative LTF consisted of repeated applications of 5-HT on days 0 and 1 of culture. EPSP amplitudes were tested before control or experimental treatments on day 0 and retested on days 3, 4, and 6. Constructs were injected into sensory neurons on day 3. **B**, Phase contrast views of sensorimotor cultures and epifluorescent views of construct expression in the same view area (control and dominant-negative form constructs) in the sensory neurons 24 h after injection. Scale bar, 50 μ m. **C**, Examples of the change in the EPSP amplitudes in L7 over time for control and experimental groups. Overexpression of dn-PKM Apl I after control treatment did not impact synaptic baseline. All experimental groups receiving repeated applications of 5-HT (2.5-HT) showed an increase in EPSP amplitude that persisted to day 6, including those that were overexpressing dn-PKM Apl I in the sensory neurons. **D**, Summary of the change in the EPSP amplitude for control and experimental groups. A two-factor ANOVA indicated a significant effect of treatment over time ($F_{(15,141)} = 66.941; p < 0.001$). Individual comparisons indicated the following: (1) overexpression of dn-PKM Apl I in the sensory neurons after control treatment (Cont + dn-PKM Apl I) had no significant effect on synaptic baseline compared with control or control + mRFP (#) on day 4 ($F_{(5,47)} = 0.004$ or 0.002 ; all $p > 0.8$) and day 6 ($F_{(5,47)} = 0.005$ or 0.011 ; all $p > 0.8$); (2) 2.5-HT produced a significant increase in synaptic strength (*) on days 3, 4, and 6 compared with those for each control group ($F_{(5,47)} = 4.689, 3.962$, and 3.474 , all $p < 0.01$ compared with Cont; $F_{(5,47)} = 5.356, 4.815$, and 3.961 , all $p < 0.01$ compared with Cont + mRFP; $F_{(5,47)} = 5.431, 4.675$, and 4.106 , all $p < 0.01$ compared with Cont + dn-PKM Apl I); (3) after 2.5-HT and overexpression of dn-PKM Apl I (2.5-HT + dn-PKM Apl I) on day 3, the change in synaptic strength remained significantly greater than for each control group (*) on days 4 and 6 ($F_{(5,47)} = 4.321$ and 3.711 , $p < 0.01$ compared with Cont; $F_{(5,47)} = 5.586$ and 4.468 , $p < 0.01$ compared with Cont + mRFP; $F_{(5,47)} = 5.407$ and 4.653 , $p < 0.01$ compared with Cont + dn-PKM Apl I), which was not significantly different from other 5-HT-treated groups on days 4 and 6 ($F_{(5,47)} = 0.061$ and 0.064 , $p > 0.7$ compared with 2.5-HT alone; $F_{(5,47)} = 0.07$ and 0.107 , $p > 0.5$ compared with 2.5-HT + mRFP).

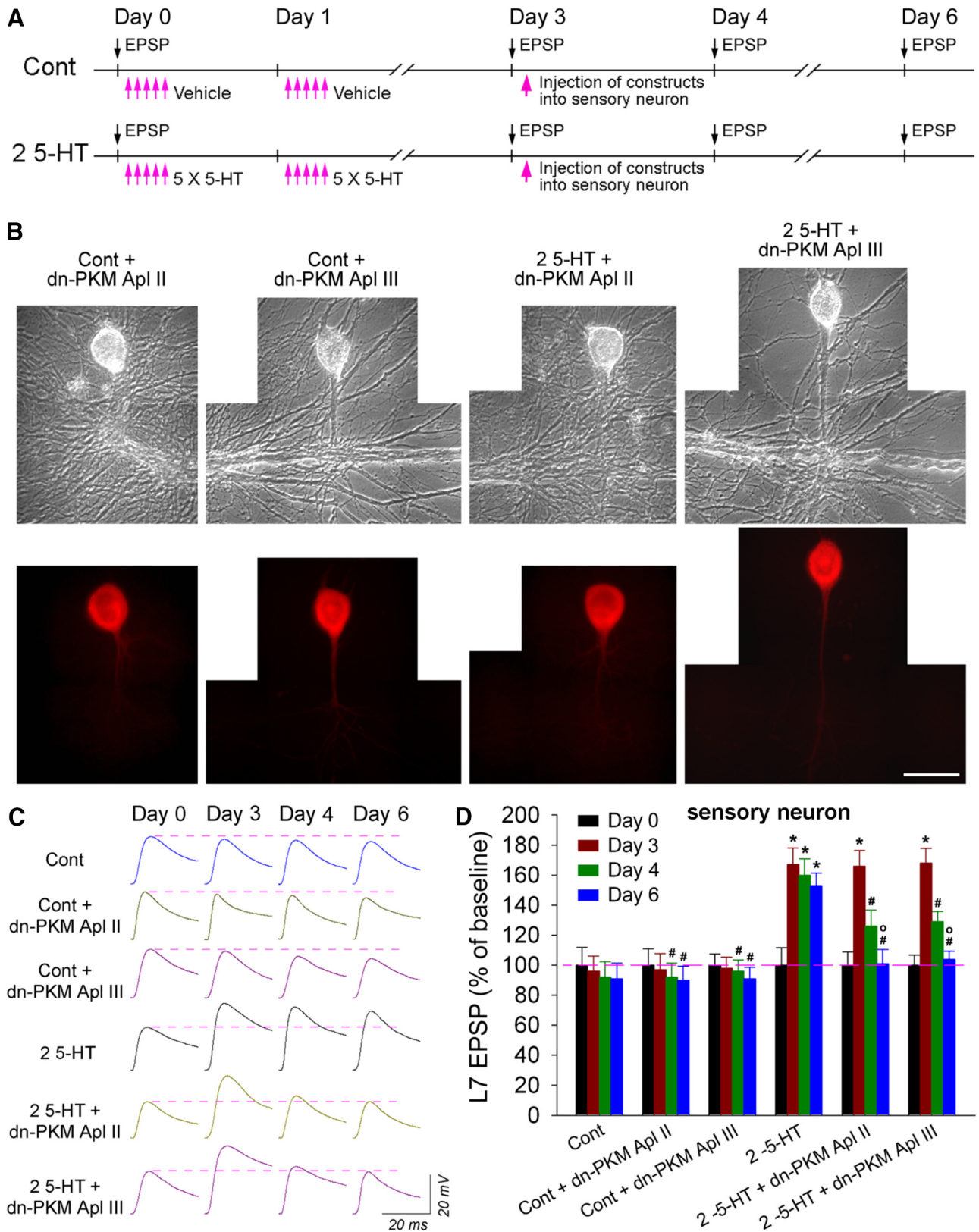


Figure 5. PKM Apl II or PKM Apl III activity in the sensory neurons is required after day 3 to maintain nonassociative LTP. **A**, Experimental protocol. Persistent nonassociative LTP was evoked by repeated applications of 5-HT on days 0 and 1. EPSP amplitudes were tested before control or experimental treatments on day 0 and were retested on days 3, 4, and 6. Constructs for dn-PKM Apl II or dn-PKM Apl III were injected into sensory neurons on day 3. **B**, Phase contrast views of sensorimotor cultures and epifluorescent views of construct expression in the same view area 24 h after injection. Scale bar, 50 μ m. **C**, Examples of the change in the EPSP amplitudes in L7 over time for control and experimental groups. Overexpression of dn-PKM Apl II or dn-PKM Apl III in the sensory neurons reversed nonassociative LTP and had no effect on synaptic baseline in control. **D**, Summary of the change in the EPSP amplitudes for control and experimental groups. A two-factor ANOVA indicates a significant effect of treatment over time ($F_{(15,144)} = 33.39; p < 0.001$). Individual comparisons indicated the following: (1) overexpression of dn-PKM Apl II or dn-PKM Apl III in the sensory neurons after control treatment (Cont + dn-PKM Apl II; Cont + dn-PKM Apl III) had no significant effect on the synaptic baseline compared with control (#) on (Figure legend continues.)

struct was shown previously to have isoform-specific effects on inhibiting physiological changes that are produced by activating their respective PKC isoforms (Manseau et al., 2001; Zhao et al., 2006; Lorenzetti et al., 2008; Hu et al., 2010).

Overexpression of dn-PKC Apl I in L7 on day 3 (Fig. 3B) did not significantly affect the maintenance of persistent nonassociative LTF (2 5-HT plus dn-PKC Apl I; $n = 10$) compared with the increases in synaptic strength evoked by 2 5-HT ($n = 7$) or by 2 5-HT plus overexpression of the control construct (2 5-HT plus eGFP; $n = 7$). Overexpression of dn-PKC Apl I (Cont plus dn-PKC Apl I; $n = 10$) in L7 on day 3 failed to affect the synaptic baseline (Fig. 3C). In contrast, the overexpression of dn-PKC Apl II ($n = 12$) or dn-PKC Apl III ($n = 10$) in L7 on day 3 resulted in a significant decline in synaptic baseline (Fig. 3C) compared with overexpression of the control construct eGFP (Cont plus eGFP; $n = 7$). Since the overexpression of dn-PKC Apl II or dn-PKC Apl III in L7 affected the synaptic baseline, we could not test their role in maintaining persistent LTF; we cannot distinguish between a reversal of the synaptic plasticity and a decrease in the basal synaptic strength (the synaptic baseline). The results suggest that PKM Apl I, but not PKC Apl I, in L7 contributes to the persistence of nonassociative LTF. In addition, PKC Apl II and PKC Apl III differ from their respective PKM counterparts in that interfering with the activity of each of the PKC isoforms affects the synaptic baseline.

Different PKM isoforms in the sensory neurons are required for maintaining persistent nonassociative LTF (Figs. 4, 5). Overexpression of dn-PKM Apl I in the sensory neurons failed to reverse persistent nonassociative LTF (2 5-HT plus dn-PKM Apl I; $n = 16$; Fig. 4). The increase in synaptic strength observed after injection of dn-PKM Apl I was comparable to the increase evoked by 2 5-HT ($n = 7$) or the increase after injection of the control construct in the sensory neurons (2 5-HT plus mRFP; $n = 8$). The increase in synaptic strength was also significantly greater than that in the Cont ($n = 6$) or after injection of the control construct (Cont plus mRFP; $n = 8$). In addition, overexpression of dn-PKM Apl I in the sensory neurons (Cont plus dn-PKM Apl I; $n = 8$) did not affect the synaptic baseline (Fig. 4C,D). Unlike the results with L7, reducing PKM Apl I activity in the sensory neurons days after 5-HT treatments does not affect the maintenance of nonassociative LTF.

Persistent nonassociative LTF was reversed after overexpressing dn-PKM Apl II (2 5-HT plus dn-PKM Apl II; $n = 10$) or dn-PKM Apl III (2 5-HT plus dn-PKM Apl III; $n = 15$) in the sensory neurons (Fig. 5). The increases in synaptic strength in both sets of cultures on day 3 before construct injection were

significantly reduced on days 4 and 6 after overexpression of dn-PKM Apl II or dn-PKM Apl III. These reversals of the increases in synaptic strength were significant compared with the increase evoked by 2 5-HT ($n = 8$). Overexpression of either dn-PKM Apl II (Cont plus dn-PKM Apl II; $n = 7$) or dn-PKM Apl III (Cont plus dn-PKM Apl III; $n = 8$) in the sensory neurons after control treatments did not affect the synaptic baseline, and synaptic strengths were comparable to those of Cont alone ($n = 6$; Fig. 5C,D). Thus, unlike the results observed with L7, in the sensory neurons PKM Apl II and PKM Apl III activity contributes to maintaining persistent nonassociative LTF, while PKM Apl I activity does not.

We also examined whether the activity of PKC Apl I, PKC Apl II, or PKC Apl III in the sensory neurons might contribute to the persistence of nonassociative LTF. Control and dominant-negative constructs were overexpressed in the sensory neurons (Fig. 6A). Overexpression of dn-PKC Apl I in the sensory neurons on day 3 (Fig. 6B) did not significantly affect the maintenance of persistent nonassociative LTF (2 5-HT plus dn-PKC Apl I; $n = 8$) compared with the increases in synaptic strength evoked by 2 5-HT ($n = 7$) or by 2 5-HT plus overexpression of the control construct (2 5-HT plus eGFP; $n = 7$). Overexpression of dn-PKC Apl I (Cont plus dn-PKC Apl I; $n = 8$) in the sensory neurons on day 3 failed to affect the synaptic baseline (Fig. 6C). In contrast, overexpression of dn-PKC Apl II ($n = 10$) or dn-PKC Apl III ($n = 10$) in the sensory neurons on day 3 resulted in a significant decline in synaptic baseline (Fig. 6C) compared with overexpression of the control construct eGFP (Cont plus eGFP; $n = 7$) or control alone. Similar to the L7 motor neuron, because the overexpression of dn-PKC Apl II or dn-PKC Apl III in the sensory neurons decreased the synaptic baseline, we could not test their role in maintaining persistent LTF. The results suggest that the activity of PKM Apl II or PKM Apl III, but not PKM Apl I or PKC Apl I, in the sensory neurons even several days after 5-HT applications, is required to sustain persistent nonassociative LTF.

Different set of cell-specific PKM isoforms maintain persistent associative LTF

Do activities of the same PKM isoforms in L7 or sensory neurons maintain persistent associative LTF produced by two pairings of activity plus 5-HT (Fig. 7A)? In L7 (Fig. 7B), overexpression of dn-PKM Apl II [2 (Tet plus 5-HT) plus dn-PKM Apl II; $n = 11$] or dn-PKM Apl III [2 (Tet plus 5-HT) plus dn-PKM Apl III; $n = 12$] on day 3 resulted in the reversal of persistent associative LTF on days 4 and 6. The change in synaptic strength was significantly reduced compared with the persistent associative LTF evoked after paired stimulation alone [2 (Tet plus 5-HT); $n = 8$] or when L7 was expressed a control construct [2 (Tet plus 5-HT) plus mRFP; $n = 8$]. In contrast, overexpression of dn-PKM Apl I in L7 [2 (Tet plus 5-HT) plus dn-PKM Apl I; $n = 10$] failed to reverse persistent associative LTF on days 4 and 6 (Fig. 7B). The level of facilitation was not significantly different from the facilitation produced by paired stimulation alone or paired stimulation plus overexpression of the control construct in L7. Thus, the PKM activities in L7 that maintain persistent associative LTF—PKM Apl II and PKM Apl III, but not PKM Apl I—are the opposite of the PKMs in L7 that maintain nonassociative LTF—PKM Apl I, but not PKM Apl II or PKM Apl III.

In the sensory neurons, overexpression of dn-PKM Apl I or dn-PKM Apl III failed to reverse persistent associative LTF produced by two pairings of activity plus 5-HT (Fig. 7C). Overexpression of dn-PKM Apl I [2 (Tet plus 5-HT) plus dn-PKM Apl I; $n = 8$] on day 3 failed to affect the facilitation evoked by two

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(Figure legend continued.) days 4 and 6 ($F_{(5,48)} = 0.002$ and 0.001 , all $p > 0.8$ for dn-PKM Apl II; $F_{(5,48)} = 0.018$ and 0.0002 , all $p > 0.7$ for dn-PKM Apl III); (2) 2 5-HT produced a significant increase in the change in synaptic strength compared with each control group (*) on days 3, 4, and 6 ($F_{(5,48)} = 3.513$, 4.190, and 4.624, all $p < 0.01$ compared with Cont; $F_{(5,48)} = 3.685$, 4.499, and 5.170, all $p < 0.01$ compared with Cont + dn-PKM Apl II; $F_{(5,48)} = 3.838$, 4.269 and 5.332, all $p < 0.01$ compared with Cont + dn-PKM Apl III); (3) after 2 5-HT and overexpression of dn-PKM Apl II (2 5-HT + dn-PKM Apl II) on day 3, the change in synaptic strength was not significantly different from Cont or Cont + dn-PKM Apl II (#) on day 4 ($F_{(5,48)} = 1.186$ or 1.267; all $p > 0.1$) and day 6 ($F_{(5,48)} = 0.132$ or 0.170; all $p > 0.5$); (4) after 2 5-HT treatments and overexpression of dn-PKM Apl III (2 5-HT + dn-PKM Apl III) on day 3, the change in synaptic strength was not significantly different from Cont or Cont + dn-PKM Apl III (#) on day 4 ($F_{(5,48)} = 1.541$ or 1.451; all $p > 0.1$) and day 6 ($F_{(5,48)} = 0.250$ or 0.286; all $p > 0.5$); (5) after 2 5-HT treatments and overexpression of dn-PKM Apl II or dn-PKM Apl III, the changes in synaptic strength were significantly smaller (o) than the change detected in 2 5-HT alone on day 6 ($F_{(5,48)} = 4.213$ or 4.412; all $p < 0.01$).

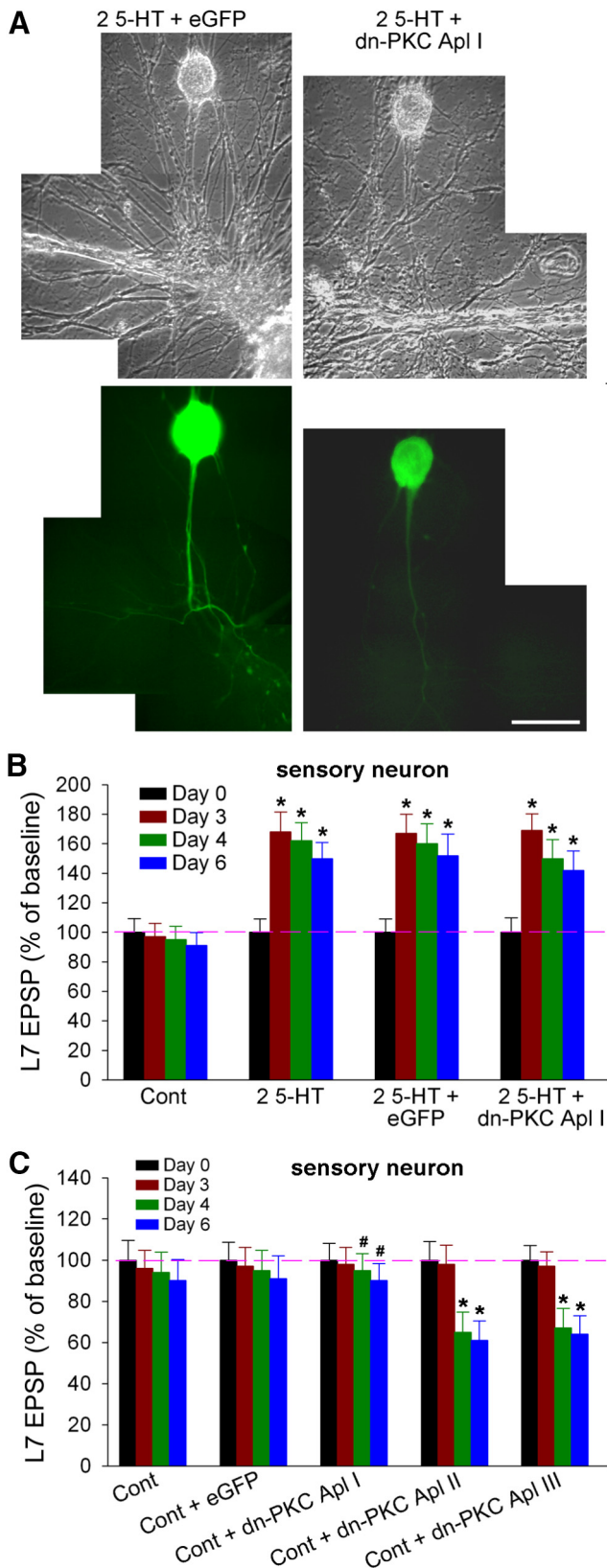


Figure 6. PKM Apl I activity in the sensory neurons is not required after day 3 to maintain nonassociative LTF. **A**, Phase contrast views of sensorimotor cultures and epifluorescent views of construct expression in the same view area (control and dominant-negative forms) in the sensory neurons 24 h after injection. Scale bar, 50 μ m. **B**, Summary of the change in the EPSP amplitude for control and experimental groups indicated that overexpression of dn-PKC Apl I in the sensory neurons (2 5-HT + dn-PKC Apl I) failed to affect persistent nonassociative LTF. A two-factor ANOVA indicated a significant effect of treatment over time ($F_{(9,72)} = 32.47$;

pairings of stimuli on days 4 and 6 compared with the change observed in 2 (Tet plus 5-HT) ($n = 6$) or following the expression of a control construct [2 (Tet plus 5-HT) plus mRFP; $n = 7$]. Overexpression of dn-PKM Apl III ($n = 8$) also failed to reverse the increase in synaptic strength produced by two pairings. In contrast, overexpression of dn-PKM Apl II ($n = 10$) in the sensory neurons reversed associative LTF on days 4 and 6. The change in synaptic strength was no longer significantly different from that of the control ($n = 6$). Thus, PKM Apl II activity in the sensory neurons maintains associative LTF, while both PKM Apl II and PKM Apl III activities in the sensory neurons maintain nonassociative LTF.

We also examined whether PKC Apl I was required for the expression of persistent associative LTF. Overexpression of dominant-negative PKC Apl I in either L7 or sensory neurons failed to interfere with associative LTF (data not shown). Dominant-negative PKC Apl I in the sensory neurons did affect a property that is characteristic of persistent associative LTF—the altered kinetics of homosynaptic depression (HSD) produced by low-frequency stimulation of the sensory neuron (Hu and Schacher, 2015). Overexpression of dominant-negative PKC Apl I, but not dominant-negative PKM Apl I or PKM Apl III, reversed HSD kinetics back to control levels (data not shown). Overexpression of dominant-negative PKM Apl II in the sensory neurons, which reversed persistent associative LTF (Fig. 7) also reversed the kinetics of HSD back to control levels (data not shown). As shown previously (Hu and Schacher, 2015), all manipulations that reversed persistent associative LTF (even overexpression of specific dominant-negative PKMs in L7) reversed the attenuated kinetics of HSD back to control levels (data not shown). Thus, PKC Apl I activity in the sensory neurons has a more restricted role in persistent associative LTF.

Activity of classical or SOL calpain is not required for the maintenance of persistent LTF but is required for the expression of persistent LTF

The *Aplysia* PKMs are generated by the action of calpains on their respective PKC isoforms (Bougie et al., 2009, 2012; Farah et al., 2016). Are calpain activities critical for the constitutive presence of PKM activities days after 5-HT applications or paired stimuli that generated the different forms of persistent plas-

$p < 0.001$). Individual comparisons indicated the following: (1) stimulation with 2 d of 5-HT applications (2 5-HT or 2 5-HT + eGFP) produced a significant increase in the change in synaptic strength compared with control (*) on day 3 ($F_{(3,24)} = 5.231$ or 4.982; all $p < 0.01$), day 4 ($F_{(3,24)} = 4.503$ or 4.333; all $p < 0.01$), and day 6 ($F_{(3,24)} = 3.535$ or 3.759; all $p < 0.01$); (2) after 5-HT treatments, overexpression of dn-PKC Apl I on day 3 in the sensory neurons had no significant effect on the change in synaptic strength, which remained significantly greater than the change observed in control (*) on days 4 and 6 ($F_{(3,24)} = 3.777$ and 3.35; all $p < 0.01$). **C**, Summary of the change in the EPSP amplitudes for various control groups with/without overexpression of dominant-negative PKCs constructs. A two-factor ANOVA indicated a significant effect of treatment over time ($F_{(12,105)} = 28.059$; $p < 0.001$). Individual comparisons indicated the following: (1) overexpression of dn-PKC Apl I in the sensory neurons on day 3 after control treatment (Cont + dn-PKC Apl I) had no significant effect on synaptic baseline compared with control or control + eGFP (#) on day 4 ($F_{(4,35)} = 0.002$ or 0.0001; all $p > 0.8$) and day 6 ($F_{(4,35)} = 0.0004$ or 0.0002; all $p > 0.9$); (2) overexpression of dn-PKC Apl II in the sensory neurons on day 3 after control treatment (Cont + dn-PKC Apl II) had a significant effect (depression) on synaptic baseline compared with control or control + eGFP (*) on day 4 ($F_{(4,35)} = 2.053$ or 2.217; all $p < 0.05$) and day 6 ($F_{(4,35)} = 1.962$ or 2.111; all $p < 0.05$); (3) overexpression of dn-PKC Apl III in the sensory neurons on day 3 after control treatment (Cont + dn-PKC Apl III) had a significant effect (depression) on synaptic baseline compared with control or control + eGFP (*) on day 4 ($F_{(4,35)} = 1.906$ or 1.974; all $p < 0.05$) and day 6 ($F_{(4,35)} = 1.967$ or 1.911; all $p < 0.05$).

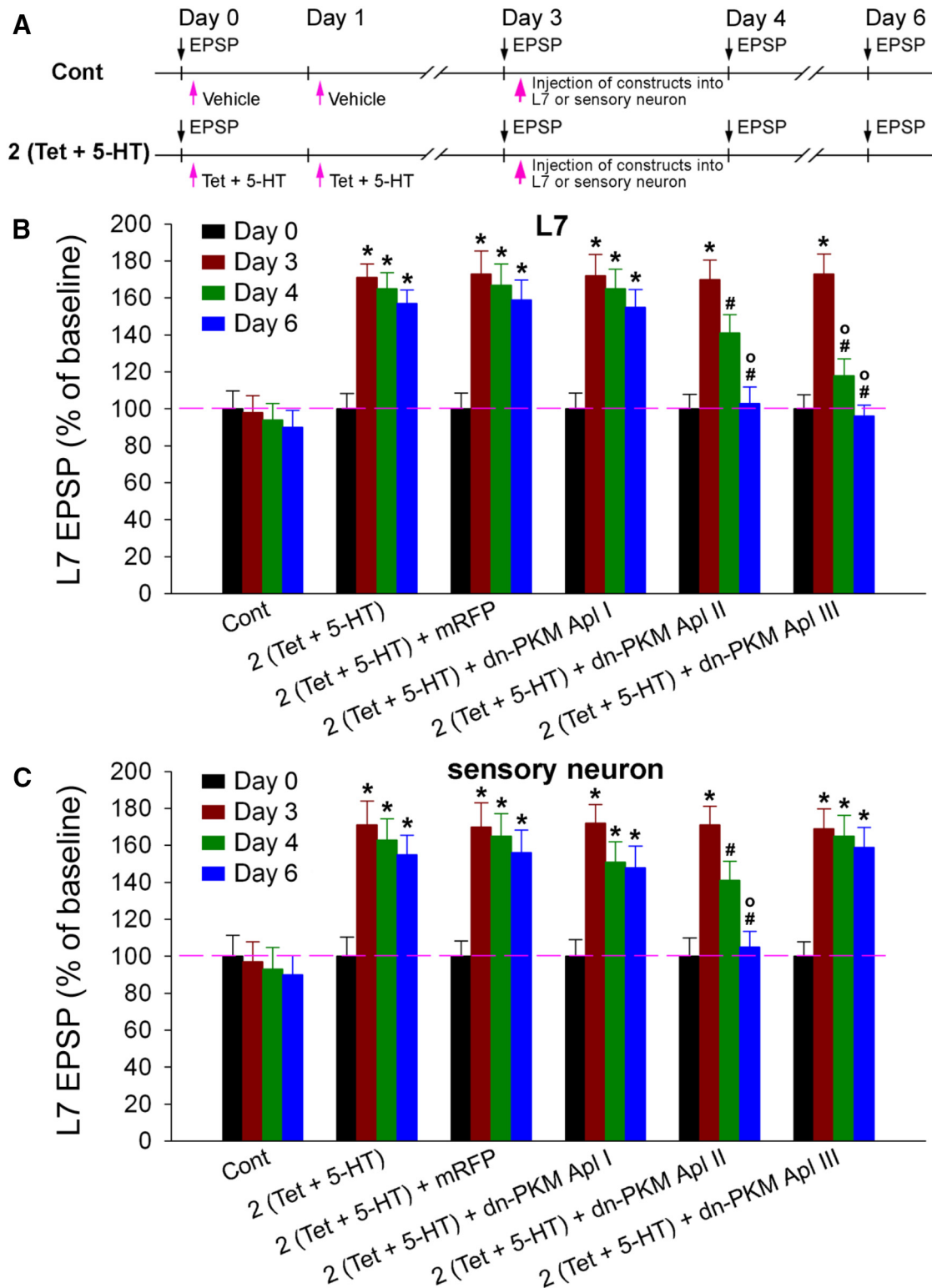


Figure 7. PKM Apl II or PKM Apl III activity in L7 or PKM Apl II activity in the sensory neurons is required to maintain persistent associative LTP. **A**, Experimental protocol for evoking persistent associative LTP consisted of pairing action potential activity in the sensory neurons (20 Hz for 2 s) with a brief application of 5-HT on days 0 and 1. EPSP amplitudes were tested before control or experimental treatments on day 0 and were retested on days 3, 4, and 6. Control or experimental constructs were injected into L7 or sensory neuron on day 3. **B**, Summary of the change in the EPSP amplitudes for control and experimental groups after overexpression of constructs in L7. A two-factor ANOVA indicated a significant effect of treatment over time ($F_{(15,150)} = 30.154$; $p < 0.001$). Individual comparisons indicated the following: (1) all groups with two pairings of stimuli produced a significant increase in the change in synaptic strength compared with control (*) on day 3 [$F_{(5,50)} = 3.687$ for 2 (Tet + 5-HT), 3.814 for 2 (Tet + 5-HT) + mRFP, 4.15 for 2 (Tet + 5-HT) + dn-PKM Apl I, 4.134 for 2 (Tet + 5-HT) + dn-PKM Apl II, and 4.567 for 2 (Tet + 5-HT) + dn-PKM Apl III; all $p < 0.01$]; (2) after two pairings of stimuli and overexpression of dn-PKM Apl I [2 (Tet + 5-HT) + dn-PKM Apl I] in L7, the change in synaptic strength remained significantly greater than that for control (*) on day 4 ($F_{(5,50)} = 4.535$; $p < 0.01$) and day 6 ($F_{(5,50)} = 5.096$; $p < 0.01$); (3) after two pairings of stimuli and overexpression of dn-PKM Apl II [2 (Tet + 5-HT) + dn-PKM Apl II] in L7, the change in synaptic strength was not significantly different from that for control (#) on days 4 and 6 ($F_{(5,50)} = 1.077$ and 0.208; all $p > 0.1$), which were significantly smaller (o) than the change detected in 2 (Tet + 5-HT) alone on day 6 ($F_{(5,50)} = 3.844$; $p < 0.01$); (4) after two pairings of stimuli and overexpression of dn-PKM Apl III (Figure legend continues.)

ticity? Dominant-negative versions of two calpain isoforms, classical or SOL, were injected into L7 or sensory neurons on day 3, the same time as the injections of dn-PKMs and dn-PKCs. Dominant-negative calpains may inhibit calpains by forming inactive dimers or by competing for binding with scaffolds or substrates. The dominant-negative classical calpain blocks both the cleavage of a FRET-sensitive reporter of *Aplysia* PKCs and the induction of plasticity during intermediate forms of facilitation (Farah et al., 2016). The persistent increases in synaptic strength evoked by repeated applications of 5-HT (nonassociative LTF) or by two pairings of activity and 5-HT (associative LTF) were unaffected by the overexpression of the dominant-negatives of either calpain isoform in L7 (Fig. 8A, C) or in the sensory neurons (Fig. 8B, D) after day 3. Overexpressing each dominant-negative calpain had no significant effect on synaptic baseline. Thus, it appears that the constitutive activations of the PKMs, days after 5-HT applications or paired stimuli that produce different forms of persistent LTF do not require the activities of classical or SOL calpain.

Are calpain activities required earlier for the expression of the different forms of persistent LTF? We injected the different dominant-negative calpains into L7 or sensory neurons on day 1, 4–5 h after the second set of stimuli (Fig. 9A, D) and examined the changes in synaptic strength on days 2, 3, and 5. Because of the earlier time of injection of the dominant-negatives, changes in the expression of LTF could be due to a block in the induction of the persistent LTF, a reversal in the initial phases of LTF from stimulation on day 0, day 1, or both.

Overexpression of dn-SOL in L7 on day 1 blocked the expression of persistent nonassociative LTF (Fig. 9B). Compared with the increase in synaptic strength produced by 2 d of 5-HT applications ($n = 6$), the synaptic strength after injection of dn-SOL ($n = 8$) was significantly smaller on days 3 and 5. In contrast, overexpression of dn-classical ($n = 8$) in L7 on day 1 did not significantly change the increase in synaptic strength of persistent nonassociative LTF on days 2, 3, and 5. Overexpression of dn-SOL ($n = 6$) or dn-classical ($n = 6$) in L7 after control treatments did not affect the synaptic baseline compared with Cont alone ($n = 6$; Fig. 9B).

Overexpression of dn-SOL in the sensory neurons on day 1 also blocked the expression of persistent nonassociative LTF (Fig.

9C). Compared with the increases in synaptic strengths produced by 2 5-HT ($n = 6$), the change in synaptic strengths after the injection of dn-SOL ($n = 8$) was significantly smaller on days 2, 3, and 5. In contrast, overexpression of dn-classical in the sensory neurons on day 1 ($n = 8$) did not significantly affect persistent nonassociative LTF. Overexpression of dn-SOL ($n = 6$) or dn-classical ($n = 6$) in the sensory neurons after control treatments did not affect the synaptic baseline compared with Cont alone ($n = 6$; Fig. 9C). Thus, the activity of SOL calpain, but not classical calpain, both in the sensory neurons and in L7, is required for the expression of persistent nonassociative LTF.

Persistent associative LTF produced by two pairings of tetanus plus 5-HT required the activity of classical calpain, but not SOL calpain, in L7 (Fig. 9E). Compared with the increases in synaptic strengths produced by two pairings alone ($n = 6$), the change in synaptic strength after overexpression of dn-classical in L7 on day 1 ($n = 8$) was significantly smaller on days 2, 3, and 5. In contrast, overexpression of dn-SOL ($n = 8$) on day 1 failed to affect the expression of persistent associative LTF.

Overexpression of dn-classical in the sensory neurons also blocked the expression of persistent associative LTF (Fig. 9F). Compared with the increases in synaptic strength produced by two pairings ($n = 6$), the change in synaptic strength after overexpression of dn-classical ($n = 8$) was significantly smaller on days 2, 3, and 5. Overexpression of dn-SOL ($n = 8$) in the sensory neurons failed to affect persistent associative LTF. Thus, the same dominant-negative constructs that failed to interfere with the maintenance of persistent plasticity when overexpression begins on day 3 now impact the expression of persistent plasticity when overexpression begins on day 1. Stimulus-dependent activation of specific calpain activities is required for the expression of different forms of persistent plasticity.

Discussion

We examined whether constitutively active PKCs, the PKMs, contribute to the maintenance of different forms of long-term plasticity starting 2 d after the stimuli that produced the plasticity. Our results suggest that repeated applications of 5-HT or pairing activity with a 5-HT application lead to the activation of specific calpains in the sensory neuron and in L7 that cleave PKCs into PKMs and contribute to the generation of cell-specific sets of PKMs. The constitutive presence of the PKMs in each neuron does not require the constitutive actions of the calpains. Specific PKM activities in each neuron contribute to the different forms of persistent plasticity. The results suggest that a timely stimulus-dependent increase in a specific calpain activity and specific sets of PKM activities in the presynaptic and postsynaptic neurons are needed for maintaining different forms of long-term synaptic plasticity at the behaviorally relevant sensorimotor synapse of *Aplysia* (Table 1, Table 2).

Cell-specific roles of PKMs in maintaining persistent LTF

Different sets of PKMs in the presynaptic and postsynaptic neuron contribute to the maintenance of each form of LTF, and, in general, there is specificity in the actions of the dominant-negatives, suggesting a specific interaction in the sensory and motor neuron with each isoform of PKM. However, both dn-PKM Apl II and dn-PKM Apl III expressed in L7 reverse associative LTF, while both dn-PKM Apl II and dn-PKM Apl III expressed in the sensory neuron reverse non-associative LTF. The similar effects of these two dominant-negatives may result because they share a similar scaffold/substrate and thus represent an example of nonisoform specificity of the dominant-negatives. Alternatively, it may result because both PKMs are inde-

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(Figure legend continued.) [2 (Tet + 5-HT) + dn-PKM Apl III] in L7, the changes in synaptic strength were not significantly different from those of control (#) on days 4 and 6 ($F_{(5,50)} = 0.551$ and 0.045 ; all $p > 0.1$), which were significantly smaller (o) than the changes detected in 2 (Tet + 5-HT) on days 4 and 6 ($F_{(5,50)} = 2.302$ and 5.10 ; $p < 0.05$ and 0.01). C, Summary of the change in the EPSP amplitudes for control and experimental groups after overexpression of constructs in the sensory neurons. A two-factor ANOVA indicated a significant effect of treatment over time ($F_{(15,117)} = 30.764$; $p < 0.001$). Individual comparisons indicated the following: (1) all groups with two pairings of stimuli produced a significant increase in the change in synaptic strength compared with control (*) on day 3 [$F_{(5,50)} = 3.446$ for 2 (Tet + 5-HT), 3.67 for 2 (Tet + 5-HT) + mRFP, 4.009 for 2 (Tet + 5-HT) + dn-PKM Apl I, 4.28 for 2 (Tet + 5-HT) + dn-PKM Apl II, 3.772 for 2 (Tet + 5-HT) + dn-PKM Apl III; all $p < 0.01$]; (2) after two pairings of stimuli and overexpression of either dn-PKM Apl I [2 (Tet + 5-HT) + dn-PKM Apl I] or dn-PKM Apl III [2 (Tet + 5-HT) + dn-PKM Apl III] in the sensory neurons, the changes in synaptic strength remained significantly greater than those for control (*) on day 4 ($F_{(5,39)} = 2.387$ or 3.660 ; $p < 0.05$ or 0.01) and day 6 ($F_{(5,39)} = 2.681$ or 3.779 ; $p < 0.05$ or 0.01); (3) after two pairings of stimuli and overexpression of dn-PKM Apl II [2 (Tet + 5-HT) + dn-PKM Apl II] in the sensory neurons, the changes in synaptic strength were not significantly different from those for control (#) on day 4 ($F_{(5,39)} = 1.810$; $p > 0.05$) and day 6 ($F_{(5,39)} = 0.209$; $p > 0.3$), which were significantly smaller (o) than the changes detected in 2 (Tet + 5-HT) on day 6 ($F_{(5,39)} = 2.192$; $p < 0.05$).

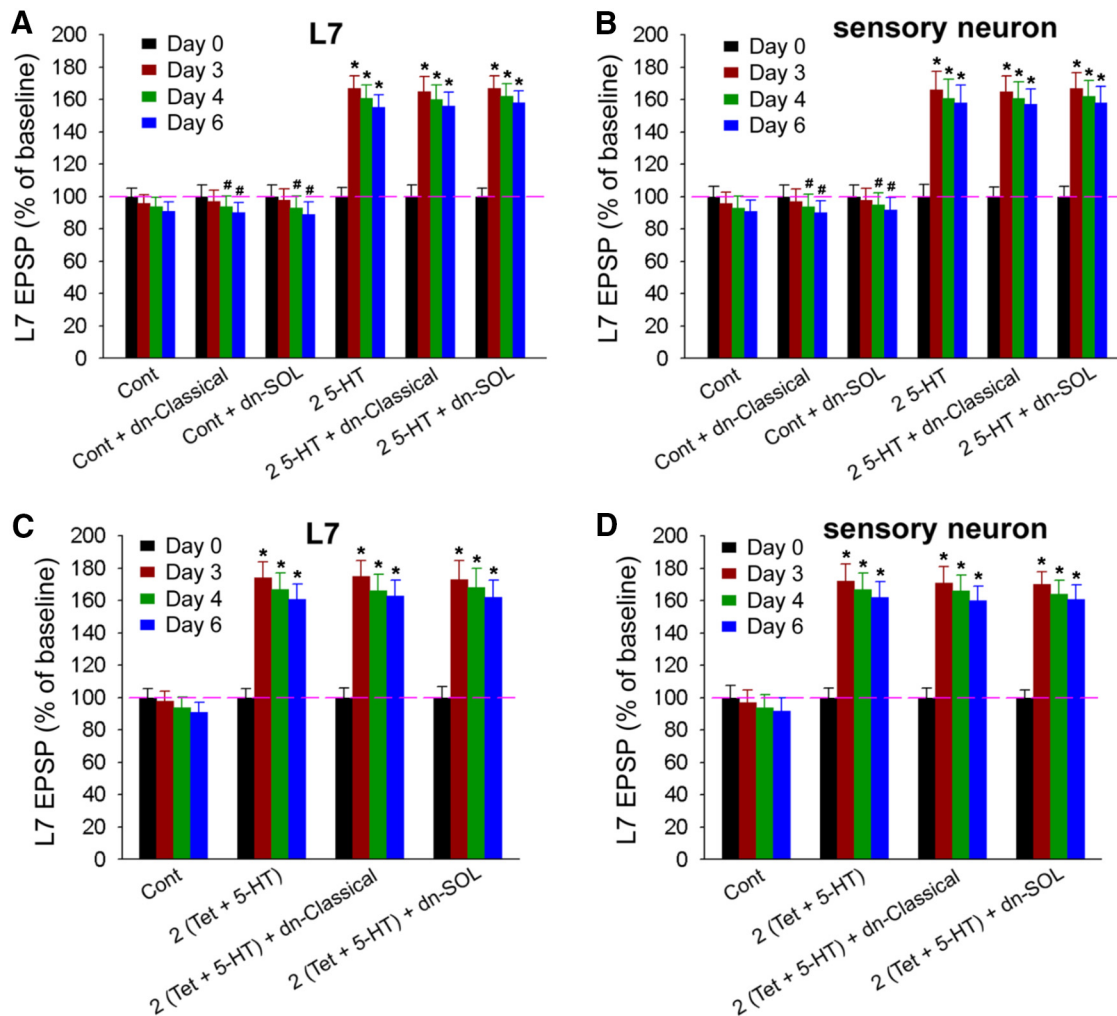


Figure 8. Classical or SOL calpain activity after day 3 is not required for maintaining persistent LTF. **A, B**, Summary of the changes in synaptic strength after control and 5-HT treatments that produced persistent nonassociative LTF with/without overexpression of dominant-negative constructs for classical or SOL calpains in L7 (**A**) or sensory neurons (**B**) on day 3. A two-factor ANOVA indicated a significant effect of treatment over time for L7 ($F_{(15,108)} = 202.9; p < 0.001$) and for sensory neuron ($F_{(15,99)} = 94.49; p < 0.001$). Individual comparisons indicated the following: (1) overexpression of dn-classical or dn-SOL on day 3 after control treatment (Cont + dn-classical or Cont + dn-SOL) had no significant effect on synaptic baseline compared with control (#) on day 4 ($F_{(5,36)} = 0.0001$ or 0.0002 , all $p > 0.9$ for L7; $F_{(5,33)} = 0.0001$ or 0.005 , $p > 0.8$ for sensory neuron) and day 6 ($F_{(5,36)} = 0.002$ or 0.007 , all $p > 0.7$ for L7; $F_{(5,33)} = 0.0001$ or 0.002 , $p > 0.8$ for sensory neuron); (2) 2.5-HT produced a significant change in synaptic strength compared with control (*) on days 3, 4, and 6 ($F_{(5,36)} = 8.871, 8.021$, and 7.526 , all $p < 0.01$ in **A**; $F_{(5,33)} = 5.804, 5.315$, and 5.464 , all $p < 0.01$ in **B**); (3) 2.5-HT plus overexpression of either dn-classical or dn-SOL on day 3 had no significant effect on the changes in synaptic strength compared to that evoked by 2.5-HT alone on day 4 ($F_{(5,36)} = 0.004$ or 0.002 , all $p > 0.7$ for L7; $F_{(5,33)} = 0.0001$ or 0.002 , all $p > 0.8$ for sensory neuron) and day 6 ($F_{(5,36)} = 0.001$ or 0.013 , all $p > 0.6$ for L7; $F_{(5,33)} = 0.001$ or 0.0001 , all $p > 0.8$ for sensory neuron), and they remained significantly greater than those for control (*) on day 4 ($F_{(5,36)} = 8.174$ or 8.84 , all $p < 0.01$ for L7; $F_{(5,33)} = 5.36$ or 5.496 , all $p < 0.01$ for sensory neuron) and day 6 ($F_{(5,36)} = 8.18$ or 8.693 , all $p < 0.01$ for L7; $F_{(5,33)} = 5.304$ or 5.464 , all $p < 0.01$ for sensory neuron). **C, D**, Summary of the changes in synaptic strength after control and stimulation treatments that produce persistent associative LTF with and without overexpression of dominant-negative constructs for classical or SOL calpains in L7 (**C**) or sensory neurons (**D**) on day 3. A two-factor ANOVA indicated a significant effect of treatment over time for L7 ($F_{(9,69)} = 59.939; p < 0.001$) and for sensory neuron ($F_{(9,66)} = 74.145; p < 0.001$). Individual comparisons indicated the following: (1) two pairings of stimuli [2(Tet + 5-HT)] produced significant changes in synaptic strength compared with control (*) on days 3, 4, and 6 ($F_{(3,23)} = 9.656, 8.603$ and 9.147 , all $p < 0.01$ in **C**; $F_{(3,22)} = 10.216, 9.845$ and 9.572 , all $p < 0.01$ in **D**); (2) after two pairings of stimuli, overexpression of either dn-classical or dn-SOL on day 3 had no significant effect on the changes in synaptic strength compared with 2(Tet + 5-HT) alone on day 4 ($F_{(3,23)} = 0.001$ or 0.002 , all $p > 0.9$ for L7; $F_{(3,22)} = 0.004$ or 0.018 , all $p > 0.5$ for sensory neuron) and day 6 ($F_{(3,23)} = 0.008$ or 0.001 , all $p > 0.7$ for L7; $F_{(3,22)} = 0.008$ or 0.001 , all $p > 0.7$ for sensory neuron), and they remained significantly greater than those for control (*) on day 4 ($F_{(3,23)} = 8.436$ or 8.874 , all $p < 0.01$ for L7; $F_{(3,22)} = 10.177$ or 9.735 , all $p < 0.01$ for sensory neuron) and day 6 ($F_{(3,23)} = 9.676$ or 9.334 , all $p < 0.01$ for L7; $F_{(3,22)} = 9.726$ or 10.148 , all $p < 0.01$ for sensory neuron).

pendently important for plasticity. Although we cannot distinguish between these two possibilities directly, the finding that only dn-PKM Apl II expressed in the sensory neuron reverses associative LTF argues against PKM Apl III and PKM Apl II competing for all forms of plasticity. While isoform-specific effects of the dn-PKMs were seen, we do not have an independent method to determine the efficacy of the dn-PKMs. Thus, we cannot rule out the possibility that the failure of a particular dominant-negative either in L7 or sensory neurons to reverse a particular form of plasticity was due to insufficient expression of the dn-PKM. If this were true, it would indicate

that distinct forms of LTF had different thresholds for PKM activity since all dn-PKMs did reverse some form of plasticity at the level of expression obtained in a specific cell (Table 1).

Each PKM activity affects critical functions that are necessary for maintaining each form of plasticity, since blocking the activity of a single isoform (in either sensory neurons or L7) leads to the reversal of a specific form of long-term plasticity. In L7, these functions might include regulating the synthesis and distribution of molecules critical for different postsynaptic functions (Trudeau and Castellucci, 1995; Zhu et al., 1997; Sherff and Carew, 2002;

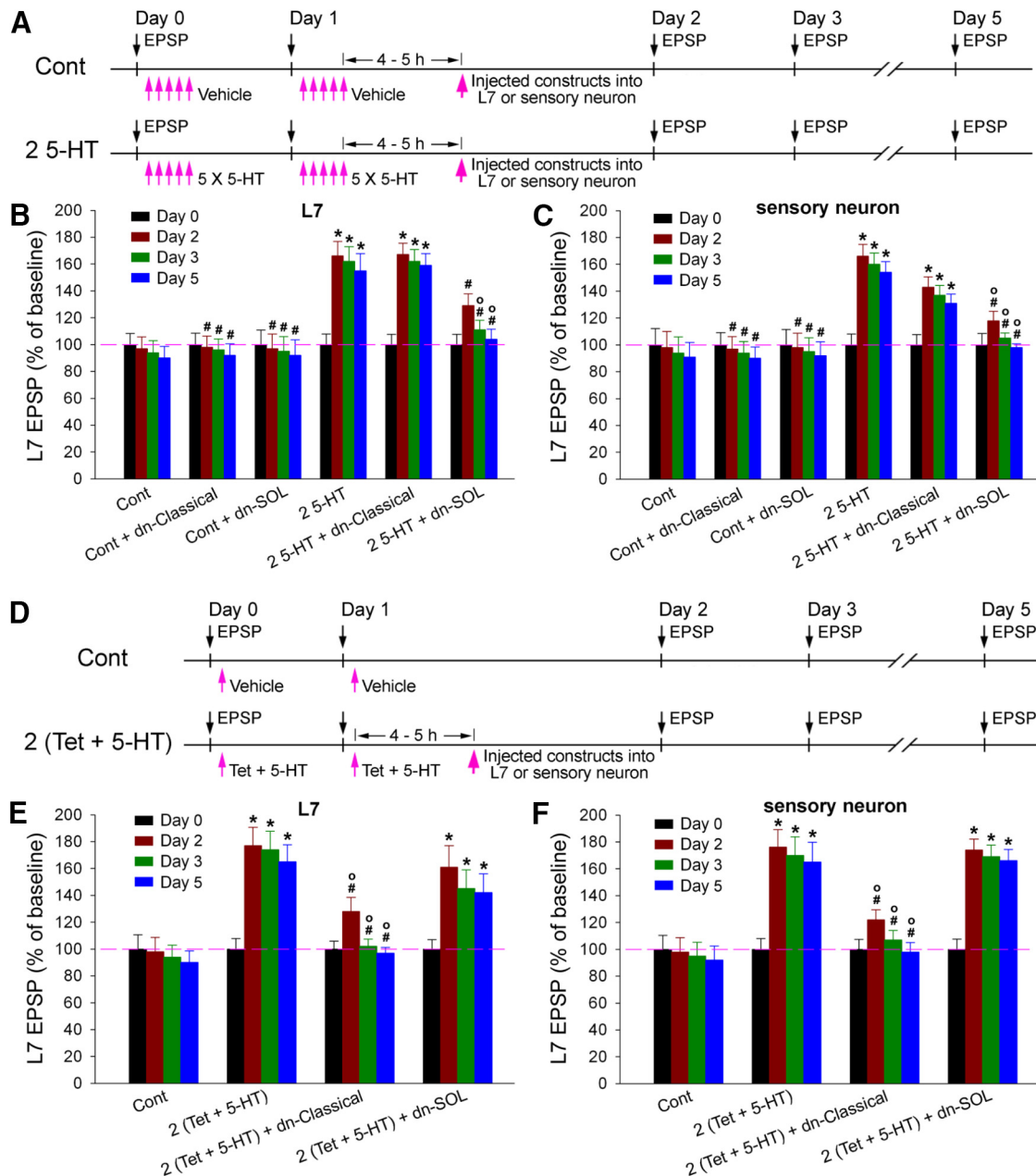


Figure 9. The SOL calpain isoform is required for the expression of persistent nonassociative LTF, while the classical calpain isoform is required for the expression of persistent associative LTF. **A**, The experimental protocol for examining the effects of overexpressing dominant-negative forms of calpain isoforms on the expression of persistent nonassociative LTF. Cultures were treated with two sets of 5-HT applications on days 0 and 1. Dominant-negative constructs of the different calpain isoforms were injected into L7 or sensory neuron 4–5 h after last application of 5-HT on day 1. EPSP amplitudes were tested before control or experimental treatments on day 0 and were retested on days 2, 3, and 5. **B, C**, Summary of the change in the EPSP amplitudes for control and stimulated groups (2 5-HT) with and without overexpression of dn-SOL or dn-classical in L7 (**B**) or sensory neurons (**C**) on day 1. A two-factor ANOVA indicated a significant effect of treatment over time for L7 ($F_{(15,102)} = 70.256; p < 0.001$) and for the sensory neurons ($F_{(15,102)} = 25.562; p < 0.001$). Individual comparisons indicated the following: (1) overexpression of dn-classical or dn-SOL in L7 on day 1 after control treatment had no significant effect on synaptic baseline compared with control (#) on day 2 ($F_{(5,34)} = 0.0003$ or 0.0001 ; all $p > 0.9$) and day 3 ($F_{(5,34)} = 0.0004$ or 0.0003 ; all $p > 0.9$) and day 5 ($F_{(5,34)} = 0.004$ or 0.002 ; all $p > 0.7$); (2) overexpression of dn-SOL or dn-classical in the sensory neurons on day 1 after control treatment had no significant effect on synaptic baseline compared with control (#) on day 2 ($F_{(5,34)} = 0.0005$ or 0.0001 ; all $p > 0.9$) and day 3 ($F_{(5,34)} = 0.0001$ or 0.002 ; all $p > 0.8$) and day 5 ($F_{(5,34)} = 0.0002$ or 0.003 ; all $p > 0.8$); (3) after stimulation to produce persistent nonassociative LTF (2 5-HT) and overexpression of dn-classical either in L7 or sensory neurons, the changes in synaptic strength remained significantly greater than control (*) on days 2, 3, and 5 ($F_{(5,34)} = 5.669, 5.738, \text{ and } 5.332$, all $p < 0.01$ in L7; $F_{(5,34)} = 2.615, 2.789, \text{ and } 2.854$, all $p < 0.05$ in the sensory neuron); (4) after stimulation to produce persistent nonassociative LTF (2 5-HT) and overexpression of dn-SOL either in L7 or sensory neurons, the changes in synaptic strength were no longer significantly different from controls (#) on days 2, 3, and 5 ($F_{(5,34)} = 1.162, 0.348, \text{ and } 0.211$, all $p > 0.05$ in L7; $F_{(5,34)} = 0.527, 0.205, \text{ and } 0.095$, all $p > 0.1$ in the sensory neuron); (5) after stimulation to produce persistent nonassociative LTF (2 5-HT) and overexpression of dn-SOL either in L7 or sensory neuron, the changes in synaptic strength were significantly smaller (o) than the change detected in 2 5-HT alone on days 3 and 5 in L7 ($F_{(5,34)} = 3.169 \text{ and } 2.922$; all $p < 0.05$), and on days 2, 3, and 5 in the sensory neuron ($F_{(5,34)} = 2.909, 4.382, \text{ and } 5.335$, all $p < 0.05$). **D**, The experimental protocol for examining the effects of overexpressing dominant-negative forms of calpain isoforms on the expression of persistent associative LTF. Cultures were treated with pairings of tetanic stimulation in the sensory neuron plus a brief 5-HT application on days 0 and 1. Dominant-negative constructs of the different calpain isoforms were injected into L7 or sensory neuron 4–5 h after the last application of 5-HT on day 1. EPSP amplitudes were tested before control or experimental treatments on day 0 and retested on days 2, 3, and 5. **E, F**, Summary of the change in the EPSP amplitudes for control and stimulated groups [2 (Tet + 5-HT)] followed by overexpression of dn-classical or dn-SOL in L7 (**E**) or sensory neurons (**F**) on day 1. A two-factor ANOVA indicated a significant effect of treatment over time for L7 ($F_{(9,72)} = 23.888; p < 0.001$) and for the sensory neurons ($F_{(9,72)} = 61.12; p < 0.001$). Individual comparisons indicated the following: (1) after stimulation to produce (Figure legend continues.)

Table 1. Overexpression of each dominant negative construct on day 3 in L7 or sensory neurons selectively affected persistent nonassociative LTF, persistent associative LTF, or synaptic baseline

	PKM Apl I	PKM Apl II	PKM Apl III	PKC Apl I	PKC Apl II	PKC Apl III
L7	Nonassociative LTF	Associative LTF	Associative LTF	NA	Synaptic baseline	Synaptic baseline
Sensory neuron	NA	Nonassociative LTF-associative LTF	Nonassociative LTF	NA	Synaptic baseline	Synaptic baseline

The effect of dominant negative PKC Apl II or PKC Apl III on persistent LTF was not examined, since each significantly affected the synaptic baseline. Cell-specific PKM/PKC isoforms are required for the maintenance of different forms of persistent LTF and synaptic baseline. NA, none affected.

Table 2. Overexpression of each dominant-negative construct on day 1 had no effect on the synaptic baseline and a selective effect on a form of persistent LTF expressed on d 3, and overexpression of the same dominant-negative constructs on day 3 had no effect on any form of persistent LTF or the synaptic baseline

	Injection on day 1		Injection on day 3	
	Classical calpain	SOL calpain	Classical calpain	SOL calpain
L7	Associative LTF	Nonassociative LTF	NA	NA
Sensory neuron	Associative LTF	Nonassociative LTF	NA	NA

Different calpain isoforms are required for the expression of different forms of persistent LTF but not for the maintenance of different forms of persistent LTF or the synaptic baseline. NA, none affected.

Antonov et al., 2003; Cai et al., 2008; Hu et al., 2015) or of the retrograde signals acting on the presynaptic sensory neuron required for enhanced synaptic function (Zhu et al., 1995; Schacher et al., 2000; Hu et al., 2010; Choi et al., 2011).

Overexpression of dn-PKMs failed to alter the synaptic baseline, while some dn-PKCs did (Table 1). One explanation for this is that the dn-PKCs and dn-PKMs interfere with separate pathways. PKCs, through their regulatory domains, may interact with substrates and/or scaffolds that are involved in sustaining synaptic baseline (Haghighi et al., 2003; Seeburg and Sheng, 2008; Diering et al., 2014; Jalil et al., 2015), whereas PKMs do not. In contrast, dn-PKM Apl I expressed in L7 reversed nonassociative LTF, but dn-PKC Apl I did not. This could be due to dn-PKM Apl I interacting through domains that are masked in dn-PKC Apl I or to dn-PKC Apl I being sequestered by interactions through their regulatory domain and thus not competing for interactions with PKM Apl I. Alternatively, dn-PKM Apl I may be expressed at higher levels than dn-PKC Apl I, and higher levels of dn-PKC Apl I expression might reverse nonassociative LTF.

In the sensory neuron, PKM Apl II and PKM Apl III activities are required for maintaining nonassociative LTF, while only PKM Apl II activity is required for maintaining associative LTF. As in L7, the PKM activities in the sensory neuron are not required to maintain the synaptic baseline, while dn-PKC Apl II and dn-PKC Apl III expression decreased the synaptic baseline. PKC Apl I activity in the sensory neurons, but not PKM Apl I, is required to maintain a property characteristic of persistent associative LTF—the altered kinetics of HSD (Hu and Schacher, 2015). The kinase activities in the presynaptic sensory neuron can

impact gene and protein expression in their cell bodies (Impey et al., 1996; Martin et al., 1997b; Patterson et al., 2001; Limbäck-Stokin et al., 2004; Lee et al., 2007; Ma et al., 2015) and at distal synaptic sites (Hu et al., 2007, 2011; Wang et al., 2009; Wan et al., 2012; Hu and Schacher, 2015), or can influence orthograde signals such as the secretion of neuropeptides, whose downstream actions are to activate additional kinases in both presynaptic and postsynaptic compartments that are required for maintaining long-term synaptic plasticity (Hu et al., 2004, 2007, 2011; Kassabov et al., 2013; Kopec et al., 2015).

Although the general role of each PKM in the different synaptic partners may be similar (regulate the activity of transcription or translation factors or modify post-translational substrates in cell compartments), the overall function of a given PKM in each partner will affect different aspects of synaptic plasticity because of the different complement or expression levels of protein substrates and scaffolds in each cell type. With associative LTF, PKM Apl II in L7 may play a critical role in the formation and maintenance of new transmitter release sites (Hu et al., 2010), while PKM Apl III in L7 may regulate the distribution and concentration of postsynaptic glutamate receptors at release sites (Villareal et al., 2009; Migues et al., 2010). PKM Apl I may regulate the distribution and concentration of postsynaptic glutamate receptors at release sites with nonassociative LTF (Trudeau and Castellucci, 1995; Zhu et al., 1997). In the sensory neuron, PKM Apl II may regulate the secretion of neuropeptides such as sensorin critical for expression of both forms of LTF (Hu et al., 2006, 2007).

Stimulus-dependent activation of specific calpain-mediated cleavage of PKCs may regulate cell-specific PKMs

How does the nature of the stimuli determine which PKM activities in the sensory neuron and L7 maintain each form of persistent plasticity? Although calpain activity is required to generate the PKMs in *Aplysia* (Sutton et al., 2004; Bougie et al., 2009, 2012; Villareal et al., 2009; Farah et al., 2016), classical and SOL calpain activities 48 h after stimuli do not participate in maintaining the presence of the PKMs required for sustaining persistent LTF. However, there are additional calpains present in *Aplysia* (Farah et al., 2016), and it is possible that a distinct calpain isoform is required for the persistence of PKM activity. It is also possible that other intracellular proteases, such as caspases, could play a role. In addition, there may be other cellular mechanisms, such as ones that prevent degradation of PKMs that may be needed to preserve the constitutive presence of the PKMs in the absence of their continual formation through protein cleavage.

Activities of specific calpain isoforms at earlier time points after the stimuli are required for the expression of each form of persistent plasticity. Blocking SOL calpain activity in the sensory neuron or L7 soon after the stimuli blocked nonassociative LTF while blocking classical calpain activity in the sensory neuron or L7 soon after the stimuli blocked associative LTF. Thus, specific stimuli induce activation of cell-specific calpain activities both at earlier time points to mediate the cleavage of appropriate PKCs to

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(Figure legend continued.) persistent associative LTF [2 (Tet + 5-HT)] and overexpression of dn-classical either in L7 or sensory neurons, the changes in synaptic strength were no longer significantly different from control (#) on days 2, 3, and 5 ($F_{(3,24)} = 0.829, 0.072, \text{ and } 0.063$, all $p > 0.1$ in L7; $F_{(3,24)} = 1.08, 0.261, \text{ and } 0.063$, all $p > 0.05$ in the sensory neuron), which were significantly smaller (o) than the changes detected in 2 (Tet + 5-HT) alone ($F_{(3,24)} = 2.277, 6.968, \text{ and } 6.736$, all $p < 0.05$ in L7; $F_{(3,24)} = 5.19, 6.857, \text{ and } 7.229$, all $p < 0.01$ in the sensory neuron); (2) after stimulation to produce persistent associative LTF [2 (Tet + 5-HT)] and overexpression of dn-SOL either in L7 or sensory neurons, the changes in synaptic strength remained significantly greater than those for control (*) on days 2, 3, and 5 ($F_{(3,24)} = 3.727, 3.456, \text{ and } 3.923$, all $p < 0.01$ in L7; $F_{(3,24)} = 10.337, 9.726, \text{ and } 8.652$, all $p < 0.01$ in the sensory neuron), which were not significantly different from the changes detected in 2 (Tet + 5-HT) alone ($F_{(3,24)} = 0.239, 1.101, \text{ and } 0.749$, all $p > 0.05$ in L7; $F_{(3,24)} = 0.01, 0.001, \text{ and } 0.0001$, all $p > 0.1$ in the sensory neuron).

produce intermediate forms of plasticity (Farah et al., 2016) and at later time points to generate the appropriate PKMs for maintaining nonassociative and associative LTF.

In summary, our results suggest that different stimuli activate different calpain isoforms that generate stimulus- and cell-specific sets of constitutively active PKMs. Different sets of constitutively active PKMs in the sensory neuron and L7 contribute to the maintenance of different forms of long-term plasticity. These results and those in the mouse hippocampus where several atypical PKCs can contribute to long-lasting long-term potentiation (Shema et al., 2011; Sacktor, 2012; Jalil et al., 2015; Tsokas et al., 2016) suggest that multiple constitutively active kinases (PKMs) in both presynaptic and postsynaptic neurons contribute to the maintenance of the long-term synaptic plasticity that underlies long-term memories.

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