MAPK Establishes a Molecular Context That Defines Effective Training Patterns for Long-Term Memory Formation

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Although the importance of spaced training trials in the formation of long-term memory (LTM) is widely appreciated, surprisingly little is known about the molecular mechanisms that support interactions between individual trials. The intertrial dynamics of ERK/MAPK activation have recently been correlated with effective training patterns for LTM. However, whether and how MAPK is required to mediate intertrial interactions remains unknown. Using a novel two-trial training pattern which induces LTM in *Aplysia*, we show that the first of two training trials recruits delayed protein synthesis-dependent nuclear MAPK activity that establishes a unique molecular context involving the recruitment of CREB kinase and *ApC/EBP* and is an essential intertrial signaling mechanism for LTM induction. These findings provide the first demonstration of a requirement for MAPK in the intertrial interactions during memory formation and suggest that the kinetics of MAPK activation following individual experiences determines effective training intervals for LTM formation.

Introduction

The formation of lasting memories significantly benefits from the temporal distribution of study sessions with intervening rest intervals (spaced versus massed training; Ebbinghaus, 1885; Cepeda et al., 2006). However, surprisingly little is known about the dynamic signaling that is important for the integration of temporally spaced trials during long-term memory (LTM) formation. In several systems, peak extracellular signal-regulated protein kinase/mitogen-activated protein kinase (MAPK) activation following individual training trials has recently been correlated with optimal intertrial intervals (ITIs) for the induction of long-term synaptic plasticity (Ajay and Bhalla, 2004) and memory (Philips et al., 2007; Pagani et al., 2009). Conversely, learning deficits in humans have been associated with a dysfunction of MAPK activation (Tidymann and Rauen, 2009). However, despite strong correlative evidence, a definitive requirement of MAPK activity in intertrial interactions during memory formation remains untested.

In the model system *Aplysia californica*, memory for sensitization, a generalized arousal induced by training shock, is sensitive to both the amount and pattern of training (Pinsker et al., 1973; Sutton et al., 2002). We recently reported a novel two-trial training pattern for the induction of LTM for sensitization of the

tail-elicited siphon withdrawal reflex (T-SWR) of *Aplysia* and identified MAPK signaling as a potential mechanism of intertrial signaling during LTM formation (Philips et al., 2007). Importantly, the two training trials had to be critically spaced: 45 min spacing intervals, but not 15 min or 1 h intervals, successfully induced LTM comparable to that induced with four spaced trials (ITI = 15 min). MAPK activation in tail sensory neurons (SNs) of the T-SWR circuit reliably correlated with the narrow two-trial training interval: MAPK is activated at 45 min following Trial 1, but is not activated at 15 min or at 1 h. Given that a single training trial is not sufficient for LTM, we hypothesized that the MAPK activated by Trial 1 is a critical factor in determining the effective intertrial interaction during two-trial LTM induction (Philips et al., 2007).

To test this hypothesis, we asked the following questions: first, what signaling determines the restricted activation of Trial 1 MAPK? Second, what signals are downstream of MAPK that create a unique temporal context in tail SNs? Finally, can we directly establish the behavioral relevance of Trial 1 MAPK activation? We found that a brief application of the neuromodulator serotonin (5HT), which is released onto SNs immediately after training shock (Marinesco and Carew, 2002), was sufficient to activate MAPK in the same temporal window as observed during behavioral training of the intact animal. Surprisingly, the transient serotonin-induced MAPK activation had four critical features: (1) it required protein synthesis, (2) it translocated to the nucleus of tail SNs, (3) it activated the CREB kinase p90rsk, and (4) it increased transcription of the CREB target, *ApC/EBP*. Finally, targeted disruption of the MAPK-dependent “molecular context” established by training Trial 1 prevented two-trial LTM formation. These findings demonstrate an essential requirement of MAPK in mediating intertrial signaling during LTM formation, and highlight the recruitment of both CREB signaling and

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gene expression within rest intervals in spaced training as potentially important MAPK-dependent signaling mechanisms that determine effective training intervals for memory formation.

Materials and Methods

Behavioral procedures. Under anesthesia, *Aplysia* (weighing 250–400 g) were surgically reduced to provide two T-TWR preparations consisting of the tail hemi-segment, ipsilateral body wall and ipsilateral pleural and pedal ganglia of the CNS interconnected by the tail (P9) nerve (Fig. 1a; Philips et al., 2011). The T-TWR was initiated by a single 0.5 s electrical stimulus to the tail test site (2–10 mA). Baseline responding (measured through a strain gauge connected to the tail) was determined through three pretests, intertest interval (ITI) = 15 min. In Figure 1, preparations were trained with two shocks (1.5 s 100 mA) to the posterior body wall through a handheld electrode or were untrained. In Figure 5, the requirement of MAPK in the intertrial interactions during two-trial LTM induction was tested using the MEK inhibitor U0126 (20 μ M in DMSO, Tocris Bioscience). U0126, or the inactive analog U0124 (20 μ M, Tocris Bioscience), was applied to the CNS chamber of behaving preparations after the second pretest. U0126/U0124 had no effect on baseline responding as assessed through comparison with a third pretest 30 min into drug incubation. Drug was washed out (5 ml ASW/min) beginning 15 min post-training Trial 1. Trial 2 was given at the 45 min intershock interval (30 min into drug washout). Post-tests were administered beginning 18–22 h post-training to assess LTM (3 post-tests, ITI = 15 min).

5HT-induced MAPK activation. MAPK activation in SNs of the VC cluster was examined in pleural-pedal ganglia isolated from anesthetized animals (each animal provided two pleural-pedal ganglia pairs). Ganglia recovered in ASW for 1.5–2 h. Molecular experiments were conducted using a counterbalanced within animal design: one ganglia pair received control treatment (ASW) and the other pair was exposed to 50 μ M 5HT for 5 min, followed by rapid washout with ASW. SN cell bodies were collected at 15, 45, or 60 min post-5HT treatment onset. Emetine (100 μ M in ASW; Sutton et al., 2001), U0126 (20 μ M in DMSO; Sharma et al., 2003) and Actinomycin D (40 μ M in ASW; Sherff and Carew, 2004) were introduced at working concentration through an initial 7 $\times$ dish volume exchange.

Western blot analysis. Lysates of tail SN cell bodies were prepared and analyzed by Western blot analysis as previously described (Sharma et al., 2003; Philips et al., 2007). The active population of MAPK in each sample was detected by probing first for phosphorylated MAPK (P-MAPK; Thr202/Tyr204, Cell Signaling Technology) and then reprobing stripped membranes for total MAPK (T-MAPK; p44/p42, Cell Signaling Technology). MAPK activation was determined using a semiquantitative analysis of the P-MAPK and T-MAPK signal in each sample and comparing P-MAPK/T-MAPK ratios obtained from 5HT-treated and control (ASW-treated) ganglia of the same animal. p90rsk phosphorylation was detected using a phospho-specific antibody (p90rsk-ser380, #9341, Cell Signaling Technology) and normalization of this signal to total β tubulin (#2146, Cell Signaling Technology). Initial characterization of the p90rsk antibody in *Aplysia* recognized a single band at $\sim$ 90 kDa that was phosphorylated downstream of MAPK (see Fig. 4a; Dalby et al., 1998; Romeo et al., 2012). p90rsk activation was described by comparing values obtained from 5HT- and ASW-treated ganglia.

Whole-mount immunofluorescent staining. Pleural-pedal ganglia were fixed (4% formaldehyde, 30% sucrose, 30 mM NaF, 3 mM NaOv in PBS) at 45 min after a 5 min 50 μ M 5HT treatment for 30 min at room temperature, then for 1 h at 4°C. Ganglia were permeabilized (4% Triton X-100, 1 h) and then quenched (1 M Glycine, 30 min). Nonspecific bind-

Figure 1. Long-term memory for sensitization of the tail-elicited tail withdrawal reflex has a narrow temporal two-trial training window. *a*, Schematic of the reduced behaving preparation used to study the tail-elicited tail withdrawal reflex (inset: representative tail withdrawal [TW] response to test stimulation [TS] as measured by a strain gauge). *b*, Summary of average 18–22 h (LTM) scores in preparations trained with two shocks spaced by 15 min (2 $\times$ 15), 45 min (2 $\times$ 45), 60 min (2 $\times$ 60), or untrained (NS). Data are presented as mean $\pm$ SEM. Asterisk indicates enhanced responding at 18–22 h in 2 $\times$ 45 trained animals over untrained controls (unpaired *t* test).

ing sites were blocked with 10% normal goat serum (diluted in 0.5% Triton X-100, 0.05% Tween-20, 30 mM NaF, 3 mM NaOv in PBS) for 1 h. Afterward, phospho- (Cell Signaling Technology, mouse, 1:200) or total-MAPK antibody (Cell Signaling Technology, rabbit, 1:100) and H2A.Z antibody (Cell Signaling Technology, rabbit, 1:200) were incubated with the ganglia for 2.5 d at 4°C. Secondary antibodies were applied at 1:200 2% goat serum for 1 h. Samples were preserved using SlowFade Gold Antifade Reagent with DAPI (Invitrogen). Confocal images through SN cell bodies were captured using the LSM 510M Confocal Microscope (Zeiss) and quantified using ImageJ by an individual blind to treatment history. SN cell bodies were identified by their location in the pleural VC cluster and their distinctive small size, ranging from 30 to 80 μ m in diameter (Walters et al., 1983b; Walters et al., 2004). Cell bodies and nuclei (using either the H2A.Z or DAPI signal as a mask) of pleural sensory neurons were manually outlined. The cytoplasm was defined by subtracting the nuclear mask from each cell body mask. Data are presented as a ratio of the nucleus to cytoplasm mean pixel intensities.

cDNA synthesis and qRT-PCR. Pleural sensory clusters were excised at 45 min after 5HT treatment and homogenized in resuspension buffer (Invitrogen). The homogenate was split to (1) synthesize cDNA (CellsDirect, Invitrogen) and (2) to confirm MAPK activation within the same sample by Western blot. Before qRT-PCR, cDNA samples were incubated with 1 μ l RNase at 37°C for 20 min to remove any residual RNA. They were then amplified (95°C 10 s, 58°C 20 s, 72°C 30 s) in a LightCycler 480 (Roche) with SYBR Green dye. The primers used in the reaction were C/EBP-F (5'-TACTCTCAACCTTCCTCAAGC-3') and C/EBP-R (5'-TGACAAATGAACAAATGCACA-3'). As a control, the amount of the ribosomal subunit S4 cDNA was detected in the same samples using primer pair S4-F (5'-GACCCTCTGGTGAAGGTGAA-3') and S4-R (5'-TGGACAGCTTCACACCTTG-3'). For quantification, the amount of ApC/EBP in each sample was first normalized to S4. Data are presented as a ratio of normalized ApC/EBP levels in the 5HT-treated side to that in the mock-treated side (C/EBP induction).

Statistical analysis. Where appropriate, a one-way ANOVA was run and is reported in the main text. Subsequent planned between group comparisons were made using a *t* test for independent means. The induction of LTM for sensitization in trained and untrained groups was additionally determined using a paired sample *t* test of the average baseline and 18–22 h responses. A within animal design was used in the molecular studies. Control and experimental values were compared using the paired samples *t* test. Due to a non-normal distribution, the Wilcoxon's matched-pairs signed rank test was used for analyzing within-group C/EBP induction. All analyses were conducted using Prism Graphpad 5. Outliers, identified as values greater than 2 SDs from the mean (a common winsorization rule; Dixon and Tukey, 1968), are indicated in the

paper and resulted in the removal of 2 of 200 data points (1%). Unless otherwise indicated, all data are expressed as mean $\pm$ SEM.

Results

Two-trial LTM induction in the tail-elicited tail withdrawal reflex of *Aplysia* has a narrow temporal window and is correlated with MAPK activation in tail SNs

We previously reported the induction of two-trial LTM in a reduced behavioral preparation of the tail-elicited tail withdrawal reflex (T-TWR; Philips et al., 2011), a significant component of which is expressed through a well characterized central tail SN–motor neuron (MN) synapse (Walters et al., 1983a; Philips et al., 2011). Here we tested the hypothesis that much of the pattern sensitivity previously observed during two-trial sensitization of the tail-elicited siphon withdrawal reflex (T-SWR; Philips et al., 2007) is mediated by the tail SNs, which provide the input pathway for both reflexes. If correct, these same SNs should impart similar pattern sensitivity during two-trial memory formation for sensitization of the T-TWR (Philips et al., 2011). T-TWR preparations were trained with two shocks (10 mA, 1.5 s) spaced by (1) 15 min (2×15), (2) 45 min (2×45), (3) 60 min (2×60), or (4) they were untrained (Fig. 1). A one-way ANOVA comparing the group scores revealed a significant effect of training on the amplitude of the T-TWR at 18–22 h post-training ($F_{(3,20)} = 6.738$, $p = 0.0025$; Fig. 1*b*). Subsequent between group comparisons of trained versus untrained animals confirmed our previous report (Philips et al., 2011) of a significant enhancement in the T-TWR response amplitude (LTM) in the group which received 2×45 training ($n = 5$, $169 \pm 20\%$), compared with untrained controls ($n = 5$, $86 \pm 12\%$, $p = 0.0072$). The T-TWR amplitude of preparations trained at either 2×15 ($n = 9$, $82 \pm 10\%$) or at 2×60 ($n = 5$, $84 \pm 24\%$) was not different from control levels ($p = 0.76$ and 0.92 , respectively). In sum, the T-TWR shares with the T-SWR (Philips et al., 2007) a narrow two-trial training interval at 45 min for LTM induction.

The narrow two-trial training interval for sensitization of the T-SWR is accompanied by the temporally restricted activation of MAPK in tail SN cell bodies at 45 min following the initial training shock (Philips et al., 2007). Consistent with this earlier observation, we found that during sensitization of the T-TWR, a single training shock results in the activation of MAPK in tail SNs at 45 min ($n = 7$, $157 \pm 15\%$ untrained phosphorylated-MAPK/total-MAPK ratio, $p = 0.0087$ paired t test, see Fig. 5*a*, ASW). Collectively, the data support the hypothesis that much of the training pattern sensitivity lies within the tail SNs which are presynaptic to both central reflex circuits, and identifies a unique opportunity to study the mechanisms underlying the temporal specificity of the two-trial training interval in the T-TWR reflex where the pre- and post-synaptic elements which contribute to the reflex are well understood.

The narrow temporal window of MAPK activation by behavioral training is also observed *in vitro* in response to exogenous 5HT application

To determine the mechanisms which underlie Trial 1 MAPK activation, we next focused on the signaling downstream of a single training shock. A single training shock results in the immediate release of the neuromodulator, 5HT, onto tail SN cell bodies and sensorimotor synapses (Marinesco and Carew, 2002; Philips et al., 2011), and brief (5 min) exposure of 5HT to the CNS can serve as a proxy for shock in (1) transiently strengthening tail SN–MN synapses (Brunelli et al., 1976; Walters et al., 1983a; Mauelshagen et al., 1996) and (2) sensitizing the T-TWR (Wal-

Figure 2. Serotonin can serve as a proxy for training shock in the restricted temporal activation of protein synthesis-dependent MAPK. *a*, A 5 min 5HT exposure to the isolated pleural-pedal ganglia induced significant MAPK activation in tail SNs at 45 min compared with paired ASW-treated control ganglia. No activation was observed at 15 min or at 1 h following the same treatment. *b*, Exposure to the protein synthesis inhibitor emetine (eme.) for 30 min before 5HT treatment prevented the 5HT-induced MAPK activation at 45 min.

ters et al., 1983a; Philips et al., 2011). Additionally, repeated training shocks or 5HT exposures give rise to sustained post-training MAPK activation in tail SNs (Sharma et al., 2003). Given these collective findings, we tested the prediction that 5HT signaling was upstream of Trial 1 MAPK activation. Pleural-pedal ganglia pairs (containing the tail SNs and SN–MN synapses) were isolated and treated with either a single 5 min application of 50 μ M 5HT or vehicle (ASW). At 15, 45, or 60 min post-onset of the treatment, tail SN cell bodies were harvested from the pleural ganglion and analyzed by Western blot for MAPK activation (Philips et al., 2007). An ANOVA of P-MAPK/T-MAPK ratios across the three groups indicated a temporally restricted activation of MAPK following 5HT treatment ($F_{(2,24)} = 4.459$, $p = 0.0226$; Fig. 2*a*). Subsequent *post hoc* analyses revealed that MAPK activation at 45 min was significantly elevated above levels observed at 15 and 60 min following 5HT exposure (LSD, $p = 0.019$ and $p = 0.030$, respectively). Within-group analyses also identified significant activation of MAPK above control levels at 45 min ($n = 11$, 1 outlier, 1.3 ± 0.08 , $p = 0.0007$, paired t test), but not at 15 min ($n = 8$, 0.99 ± 0.13 , $p = 0.683$) or 60 min ($n = 8$, 1 outlier, 1.0 ± 0.08 , $p = 0.627$). 5HT-induced MAPK activation required MEK activity, since activation at 45 min was completely disrupted in the presence of the MEK inhibitor, U0126 ($n = 5$, $92 \pm 10\%$ 5HT-/ASW-treated, $p = 0.434$ paired t test, data not shown). The similar temporal profile of MAPK activation following training shock (Philips et al., 2007) and 5HT treatment strongly supports the hypothesis that MAPK is activated by the 5HT released during the initial training shock (Trial 1).

The delayed temporal window of Trial 1 MAPK activation requires translation but not transcription

Having established that 5HT treatment induces the same temporal profile of MAPK activation as observed following training shock, we next explored the mechanisms of the delayed activation. We have previously shown that a sustained (1–3 h) MAPK activation develops in tail SNs following spaced training (4 or 5 spaced training shocks or pulses of 5HT; Sharma et al., 2003) and is necessary for LTM formation (Shobe et al., 2005). Induction of the 5HT-dependent sustained MAPK activity requires protein synthesis, but not new gene transcription (Hu et al., 2004, 2006). To examine the mechanistic correspondence between repeated trial sustained MAPK and Trial 1 MAPK, we next asked whether

the delayed and transient activation of MAPK by 5HT also requires new protein synthesis. Isolated pleural-pedal ganglia were exposed to 5HT in the presence or absence of the protein synthesis inhibitor emetine (100 μ M). Emetine treatment had no effect on basal levels of phosphorylated MAPK ($n = 8$, 1.13 $\pm$ 0.21 P-MAPK/T-MAPK ratio ASW/ASW-emetine, $p = 0.909$, data not shown). However, emetine exposure to the CNS for 30 min before 5HT application completely blocked 5HT-induced delayed MAPK activation ($n = 19$, 5HT: 1.64 $\pm$ 0.16 P-MAPK/T-MAPK ratio, 5HT-emetine: 1.19 $\pm$ 0.07, $p = 0.003$ paired t test, Fig. 2*b*). This surprising requirement for transient MAPK activation after a single training trial implies that protein synthesis is engaged much earlier in training than previously appreciated. Similar to repeated trial MAPK activation (Sharma et al., 2003), treatment with the transcriptional inhibitor Actinomycin D (50 μ g/ml) for 1 h before ASW or 5HT exposure did not disrupt Trial 1 MAPK activation ($n = 12$, 5HT/ASW: 1.52 $\pm$ 0.23, $p = 0.029$). Collectively these data show that single-trial MAPK activation at 45 min requires new protein synthesis, but not transcription. The dependence of Trial 1 MAPK activation on protein synthesis may be sufficient to account for the delay in its activation and therefore also sufficient to account for the timing of the LTM permissive two-trial training interval.

Single-trial 5HT exposure drives the nuclear translocation of MAPK

MAPK is significantly phosphorylated in isolated SN cell bodies at 45 min following a single training trial, but not at 15 min or at 1 h. This phosphorylation could, in principle, support the translocation of MAPK to the nucleus to participate in LTM induction (Turjanski et al., 2007). To explore the subcellular location (nuclear vs cytoplasmic) of activated MAPK at 45 min, we next examined pleural-pedal ganglia pairs ($n = 8$) treated with 5HT or ASW, fixed at 45 min and immunostained for P-MAPK. DAPI was used to label nuclei. Tail SN cell bodies were then imaged by confocal microscopy (Fig. 3). 5HT significantly increased the nuclear:cytoplasmic ratio of P-MAPK in tail SNs at 45 min (5HT: mean pixel intensity $\pm$ SEM, 0.84 $\pm$ 0.03, ASW: 0.65 $\pm$ 0.03, $p = 0.038$; Fig. 3). Cytoplasmic levels of P-MAPK were also increased by 5HT treatment (5HT: 86 $\pm$ 3 mean pixel intensity, ASW: 52 $\pm$ 2, $p < 0.001$). Additionally, we examined T-MAPK immunoreactivity in pleural-pedal ganglia treated with either 5HT or ASW. As with P-MAPK labeling, we found a significant increase in the nuclear:cytoplasmic ratio of T-MAPK immunostaining in tail SN somata at 45 min post 5HT treatment ($n = 6$, 5HT: 0.66 $\pm$ 0.02, ASW: 0.53 $\pm$ 0.02, $p = 0.034$, data not shown). Collectively, these data support a model in which a single training trial, via the release of 5HT, recruits the temporally restricted nuclear translocation of activated MAPK in tail SNs at 45 min.

Trial 1-induced MAPK transiently activates the CREB kinase p90rsk and increases transcript levels of the immediate early gene *ApC/EBP*

The phosphorylation and subsequent nuclear translocation of MAPK is thought to be an essential step in the activation of the transcription factor CREB, and CREB-dependent immediate early gene transcription necessary for LTM induction (Yin et al.,

Figure 3. Trial 1 MAPK translocates to the nucleus of tail sensory neurons. ASW- and 5HT-treated pleural ganglia were fixed beginning 45 min after the onset of treatment and labeled for phosphorylated MAPK (P-MAPK). DAPI labeling identifies cell nuclei. Left, Representative confocal microscopy images through the VC cluster of sensory neurons. Arrowheads identify cells with representative nuclear translocation. Scale bar, 50 μ m. Right, Summary analysis of nuclear/cytoplasmic ratio of P-MAPK immunofluorescence. Asterisk indicates a significant increase in the nuclear/cytoplasmic ratio at 45 min post-5HT. Number of VC clusters/total number of cells analyzed are indicated.

1994; Guzowski and McGaugh, 1997; Sharma and Carew, 2004; Lee et al., 2008). MAPK is known to activate CREB indirectly through the cytoplasm-localized kinase p90rsk (RSK1/2; Arthur et al., 2004), and p90rsk phosphorylation (activation) is correlated with MAPK activation during learning (Sindreu et al., 2007). We explored the potential recruitment of p90rsk following 5HT treatment and found that its phosphorylation is significantly increased above control levels in SNs at 45 min following a single 5 min 5HT treatment ($n = 14$, 5HT: 1.1 $\pm$ 0.08 phosphorylated p90rsk/ β tubulin ratio; ASW: 0.8 $\pm$ 0.09, $p = 0.001$ paired t test; Fig. 4*a*). Moreover, 5HT-induced p-p90rsk was completely disrupted by U0126, demonstrating that it is downstream of MAPK activity (U0126: $n = 6$, 0.86 $\pm$ 0.07 5HT/ASW ratio, $p = 0.063$, U0124: $n = 4$, 2.1 $\pm$ 0.8, $p = 0.04$). Finally, the activation of p90rsk was transient and was no longer observed at 1 h following 5HT treatment ($n = 7$, 1.3 $\pm$ 0.4 5HT/ASW ratio, $p = 0.77$ paired t test, NS, data not shown). These data show that Trial 1 MAPK signals through a transient activation of the CREB kinase, p90rsk, at the 45 min two-trial training window.

We next explored the potential impact of MAPK-dependent p90rsk activation and nuclear MAPK activity, which can promote CREB-mediated transcription, by examining a primary downstream target of CREB, the immediate early gene *ApC/EBP*. *C/EBP* is critical for long-term memory formation in *Aplysia* and other systems (Alberini et al., 1994; Taubenfeld et al., 2001). Using quantitative RT-PCR we examined whether transcript levels of *ApC/EBP* were differentially affected in the permissive time window 45 min following a single 5HT treatment. We found that *ApC/EBP* message levels were significantly increased at 45 min after 5HT relative to a control, the S4 ribosomal subunit ($n = 10$, median $\pm$ IQR, 1.6 $\pm$ 2.7, $W = 7$, $p = 0.04$, Wilcoxon matched-pairs signed rank test; Figure 4*b*). In a parallel analysis of the same samples, we confirmed MAPK activation ($p = 0.001$). *ApC/EBP* induction required MAPK activation since it was significantly disrupted by U0126 treatment (U0126: $n = 8$, median $\pm$ IQR, 0.95 $\pm$ 0.4 control levels, $W = 10$, $p = 0.55$, U0124: $n = 7$, 1.25 $\pm$ 1.3, $W = -28$, $p = 0.02$). Finally, *ApC/EBP* induction was not observed at 1 h following 5HT treatment ($n = 6$, median $\pm$ IQR, 1.0 $\pm$ 1.25 control levels, $W = -1$, $p > 0.99$, data not shown). Thus, MAPK-dependent *ApC/EBP* induction is observed during the 45 min training window, but this induction is transient and is not observed at 1 h, in the absence of MAPK activation. Collectively, these data lend strong support to the hypothesis that

Figure 4. Trial 1 MAPK activates the CREB kinase p90rsk and increases transcription of the immediate early gene *ApC/EBP*. **a**, Top, Representative image of phosphorylated p90rsk (p-p90rsk) levels in ASW- and 5HT-treated SN clusters at 45 min. Bottom, Summary analysis of normalized p-p90rsk levels observed in ASW- and 5HT-treated SNs. Exposure to the MEK inhibitor, U0126, disrupted the observed 5HT-induced increase in p-p90rsk. Treatment with the inactive analog, U0124, had no significant effect. Data in histograms are presented as mean $\pm$ SEM. **b**, qRT-PCR was performed to determine transcript levels of the immediate early gene *ApC/EBP* and the ribosomal *S4* subunit. Levels of *ApC/EBP* (normalized to levels of *S4*) were increased by 5HT. U0126, but not U0124, disrupted the 5HT-induced *ApC/EBP* induction. Asterisks indicate significant within-group activation. Data in histograms are presented as median $\pm$ interquartile range.

MAPK is upstream of the transient induction of CREB-mediated gene expression following a single training trial.

Trial 1-induced MAPK activity is required for two-trial LTM

While peak levels of activated MAPK have been correlated with optimal training intervals for the induction of long-term plasticity and memory during spaced training (Ajay and Bhalla, 2004; Philips et al., 2007; Pagani et al., 2009), the essential requirement for MAPK signaling in the same intertrial interactions during plasticity and memory formation has not yet been demonstrated. We have previously shown that U0126 (but not the inactive analog U0124) disrupts the formation of repeated-trial LTM in a reduced behaving preparation of the T-SWR (Sharma et al., 2003). Using a similar treatment strategy (CNS exposure to drug before, during and for 1 h after training), we first established that MAPK activation was required for the induction of two-trial LTM (U0124: $n = 4$, $204 \pm 51\%$ baseline T-TWR amplitude, U0126: $n = 4$, $68 \pm 9\%$ baseline T-TWR amplitude, $p = 0.04$ unpaired t test, data not shown). This result confirms that MAPK activity during or after training (or both) is required for two-trial LTM.

Having found that Trial 1 MAPK translocates to the nucleus and promotes CREB transcriptional activity by 45 min (Figs. 3, 4), we next specifically tested the hypothesis that the MAPK activation observed following a single training trial (Fig. 5a, ASW) is necessary to establish the permissive ITI at 45 min during two-trial LTM induction. We restricted U0126 exposure to disrupt

Figure 5. Trial 1 MAPK is necessary for the intertrial interaction during induction of LTM. **a**, Experimental strategy to selectively disrupt MAPK activation during the initial training shock (ASW histogram), while leaving intact the potential for MAPK activation by the second shock during two-trial training. I, CNS exposure to U0126 for 40 min before, during and 15 min after the administration of a single training shock to behaving T-TWR preparations was sufficient to disrupt Trial 1 MAPK activation in tail SNs (ASW vs I in summary histogram, right). II, A 30 min drug washout following the same treatment in I (55 min U0126 exposure, no training), was sufficient to restore MEK activity as demonstrated by the ability of a single shock at this time point to activate MAPK at 45 min postshock. **b**, The specific disruption of Trial 1-induced MAPK activation during two-trial learning prevents LTM formation. Asterisk indicates a significant difference in the between group comparison. Data are mean $\pm$ SEM.

only MAPK activation by training Trial 1, but not activation by subsequent training (Trial 2). U0124/U0126 was applied to the CNS of reduced T-TWR preparations 40 min before training, during the establishment of baseline responding, and remained until 15 min after Trial 1 (Fig. 5a). This treatment significantly disrupted Trial 1 MAPK activation observed at 45 min ($n = 5$, $82 \pm 17\%$ control; Fig. 5a,I). Additionally, the inhibition of MEK could be readily reversed before the temporal window for the second training shock at 45 min, as evidenced by the restoration of MAPK activation when a single shock was administered to T-TWR preparations following 30 min of drug washout ($n = 6$, $147 \pm 16\%$ control, $p = 0.044$; Fig. 5a,II). Thus, treatment with U0126 until 15 min post-training Trial 1, permits us to specifically test the requirement for MAPK activation by Trial 1 in two-trial LTM induction, without disturbing the ability of the second training shock (Trial 2) to activate MAPK. Using this experimental strategy (Fig. 5a), in control experiments we observed significant LTM at 18–22 h after training in the presence of U0124 ($n = 7$, $176 \pm 26\%$ baseline T-TWR amplitude, $p = 0.012$ paired t test, Fig. 5b). However, LTM induction was significantly disrupted by the specific elimination of Trial 1 MAPK activation (U0126: $n = 7$, $119 \pm 21\%$ baseline, $p = 0.658$). These data establish that MAPK activity recruited by the initial training shock is required for the intertrial interaction during two-trial LTM induction.

Discussion

A model summarizing our findings is shown in Figure 6. Two-trial LTM for sensitization of both the T-SWR (Philips et al., 2007) and the T-TWR (Fig. 1) shares a narrow permissive training interval at 45 min. This observation is consistent with the hypothesis that much of the pattern sensitivity during memory formation occurs within the common input pathway to both reflexes, the tail SNs. Brief treatment of the CNS with 5HT, which can substitute for sensitization training in the T-TWR (Walters et al., 1983a; Philips et al., 2011), was sufficient to mimic the training shock-induced delayed and restricted activation of MAPK observed in tail SNs at the 45 min permissive window for two-trial LTM induction (Philips et al., 2007). Unexpectedly, we found that MAPK activation induced by Trial 1 required protein synthesis which may, in part, explain the temporal delay in MAPK activation observed during training. We also found that Trial 1 MAPK translocated to the nucleus of tail SNs by 45 min and established a unique and transient molecular context, which included activation of the CREB kinase p90rsk and increased expression of the CREB target and IEG, *ApC/EBP*. Finally, we established the behavioral relevance of the Trial 1 MAPK-dependent “molecular context,” which was required for the intertrial interaction during two-trial LTM induction. Thus we demonstrate an essential requirement for MAPK activity in the intertrial interactions during pattern-sensitive LTM formation and provide a testable model in many other systems that the recruitment and nuclear signaling of MAPK (perhaps surprisingly early) during learning trials may ultimately determine effective training intervals during memory formation.

The kinetics of Trial 1 MAPK activation establish the two-trial training interval that is effective in LTM induction

The two-trial training interval for LTM induction in both the T-SWR and the T-TWR is surprisingly narrow, restricted to 45 min (but not 15 min or 1 h) following an initial training shock (Fig. 1; Sutton et al., 2002; Philips et al., 2007). Given that MAPK activity is a necessary component of the intertrial signaling during two-trial learning (Fig. 5), and is not activated in the somatic compartment of tail SNs of the reflex circuit until 45 min, the delay in MAPK activation may be sufficient to explain the failure of the 15 min training interval to induce LTM. It is not until 45 min, when the MAPK-dependent molecular context in tail SNs has been established, that a second training shock can support LTM formation (Fig. 6). Strikingly, following both tail shock (Philips et al., 2007) and 5HT treatment (Fig. 2), single-trial MAPK activation is inactivated by 1 h. This

Figure 6. A model depicting how MAPK establishes a molecular context that defines effective training intervals for LTM. A single training shock (TS) releases 5HT onto tail SNs and sensorimotor synapses (Marinesco and Carew, 2002; Philips et al., 2011). 5HT acts at SN membranes through a receptor-coupled adenylate cyclase to increase levels of cAMP and activate PKA in sensory neuron somata and synaptic terminals (Bacskai et al., 1993; Muller and Carew, 1998). cAMP/PKA activation is transient and is inactivated within 15 min following TS. MAPK activation by the same TS is delayed, and appears in the cell body and nucleus at 45 min, but not at 15 min or 1 h. Trial 1 MAPK activation requires protein synthesis which may account, at least in part, for its delayed activation. Trial 1 MAPK activity establishes a unique molecular context within tail SNs by 45 min that is required for two-trial LTM formation. By 45 min, MAPK has activated the CREB pathway as observed in the increased phosphorylation of the CREB kinase p90rsk and increased *ApC/EBP* expression. By 1 h, the MAPK-dependent molecular context has ended. Although required for LTM, this MAPK-dependent molecular context is not sufficient for LTM and must interact with additional signaling from a second TS at 45 min to induce LTM (two-trial LTM induction). The necessary signaling provided by the second TS during two-trial LTM induction may be the recruitment of additional 5HT release, PKA activity and the PKA-dependent activation of the *ApC/EBP*/APAF complex which is sufficient to drive long-term synaptic facilitation at SN–MN synapses (Lee et al., 2006). The delayed onset of the MAPK-dependent molecular context may be sufficient to explain the failure of the short (15 min) training interval to support LTM formation. Longer (1 h) training intervals may similarly fail to support LTM induction because the required MAPK signaling and MAPK-dependent gene expression has ended by 1 h.

rapid inactivation of MAPK, and of the MAPK-dependent recruitment of CREB kinase and *ApC/EBP* expression, by 1 h may account for the failure of this longer training interval in two-trial LTM induction (Sutton et al., 2002; Philips et al., 2007). Importantly, the kinetics for the activation of MAPK in the cell body and nuclei of tail SNs following an initial training experience appears to determine the delayed (45 min) two-trial training interval for LTM induction and is consistent with the correlations of intertrial MAPK activation to successful training intervals during LTM formation in other systems (Pagani et al., 2009).

We studied possible mechanisms for the activation of Trial 1 MAPK and found that it required protein synthesis (Fig. 2). Intriguingly, rapid rates of protein synthesis and degradation have recently been implicated in the neuronal processing underlying

synaptic facilitation of SN–MN synapses that develops across repeated spaced, but not massed, applications of 5HT (Naqib et al., 2011). The sustained activation of nuclear MAPK following five repeated spaced training shocks or spaced pulses of 5HT (Sharma et al., 2003) appears to be initiated in the presynaptic terminals of SN–MN synapses and requires the synthesis and local synaptic signaling of the neurotrophin-like peptide sensorin (Hu et al., 2004; Wang et al., 2009). Given (1) that Trial 1 MAPK activation requires protein synthesis and (2) that protein synthesis is increased within minutes following brief 5HT treatment to isolated SN neurites in culture (Casadio et al., 1999; Si et al., 2003), an intriguing possibility is that a sensorin-dependent signal in the synapse may be upstream of Trial 1 MAPK activation. Consistent with this idea, in a preliminary investigation we observed that 5HT-induced MAPK activation at 45 min could be disrupted with a blocking antibody against sensorin that antagonizes the autocrine signaling of sensorin at tail SNs. Collectively, the data suggest that the timing of the MAPK-dependent two-trial training interval is a reflection of the dependence of Trial 1 MAPK activation on new protein synthesis (perhaps the local synaptic translation of sensorin).

Finally, the recruitment of a second, delayed phase of MAPK activation following training has been shown to be important for the development of long-term memory following both multiple-trial spaced (Atkins et al., 1998; Shobe et al., 2005) and single-trial (Cammarota et al., 2000; Michel et al., 2011) training patterns. In *Aplysia*, we previously reported that the delayed MAPK activation that follows spaced training requires protein synthesis, but not transcription (Shobe et al., 2005 and unpublished observations), similar to the present observations for intertrial MAPK activation. Both the extent to which the mechanisms responsible for the delayed activation of MAPK in these two phases of memory formation are similar or different, as well as the potential relationship of these two phases, remain important questions for further study. Importantly, the delayed recruitment of MAPK during learning (i.e., following single training trials that are subthreshold for LTM induction) has now been described in several systems (Philips et al., 2007; Pagani et al., 2009), and although it has received less attention than post-training MAPK signaling, it appears to be essential in determining effective training intervals across several species.

CREB and *ApC/EBP* are recruited as part of a MAPK-dependent molecular context important for intertrial signaling during LTM formation

The temporal correlation between the appearance of activated MAPK in the cell bodies of tail SNs (cytoplasm and nucleus) and the permissive two-trial training interval suggests an important role for MAPK activity in this specific cellular compartment for intertrial signaling during LTM induction. In support of this notion, we found that Trial 1 MAPK established a molecular context of CREB signaling that included *ApC/EBP* induction (Fig. 4), an essential requirement for LTM formation. Given that a single training shock is not sufficient for LTM induction, the recruitment of nuclear MAPK translocation, CREB kinase and *ApC/EBP* is quite surprising, and provides initial experimental evidence suggesting novel roles for CREB and *ApC/EBP* in the intertrial signaling important for LTM formation over spaced training trials. Consistent with this notion, Parsons and Davis (2012) recently reported the recruitment at 1 h of a transient CREB phosphorylation in the amygdala of rats given a single training trial which, in itself, was subthreshold for the induction of STM and LTM for a form of fear memory. Additionally, Silva and

colleagues (1998) previously suggested that minimal effective spaced training intervals may be determined by the time required to recruit CREB and to complete a round of CREB-dependent gene expression before subsequent training trials could effectively recruit additional rounds of transcription. Finally, our findings are also consistent with the intriguing hypothesis, that since many immediate early genes (IEGs) code for transcription factors, they may be recruited during learning as agents of metaplasticity (Clayton, 2000; Kaczmarek, 2000; Guzowski, 2002). From this perspective, IEG induction following an initial experience would serve to “prime” the transcriptional response during a subsequent experience to support the transcriptional requirements of LTM induction (Guzowski, 2002). Consistent with this notion, the overexpression of *ApC/EBP* in *Aplysia* SNs is not sufficient to support the induction of long-term facilitation at sensorimotor synapses, but must be combined with a brief 5HT exposure (Lee et al., 2001). Indeed, a MAPK-dependent *ApC/EBP* induction by Trial 1 may interact with 5HT release following Trial 2 to support two-trial LTM formation. Importantly, the role of IEGs in intertrial signaling can now be directly examined using the present two-trial training paradigm we have developed.

Two-trial training patterns provide a unique opportunity to study the spacing effect in LTM formation

At a broader level, this work demonstrates the unique advantages provided by simple training patterns for the study of the mechanisms underlying the temporal specificity of intertrial intervals during LTM formation. Since the pioneering work of Ebbinghaus (1885) it has been widely appreciated that the spacing of repeated training trials benefits memory retention. But the mechanisms that determine the benefit of specific training intervals are completely unknown. In part, this is due to the common practice of training with multiple spaced training trials, which makes it inherently difficult to determine the contribution of each individual trial to the development of the final memory. A recent seminal study by Zhang et al. (2012) used a computer model, which incorporated basic knowledge of the kinetics of PKA and MAPK activation following a single training trial in the tail SNs of *Aplysia*, to predict effective five-trial spaced training patterns that would result in the maximal coincident recruitment of PKA and MAPK signaling. Computer simulations, followed by experimental validation, identified “enhanced” training patterns over the standard regularly spaced protocol for the induction of long-term synaptic facilitation and LTM that were surprisingly non-intuitive and shared in common the feature that they were not regularly spaced in time (Zhang et al., 2012). Together with recent findings that just two appropriately spaced training trials can support LTM induction in *Aplysia* (Philips et al., 2007) and rats (Parsons and Davis, 2012), we suggest that an optimal strategy for understanding how spaced experiences interact to support LTM induction is to begin with two simple questions: First, what signaling is established by an initial experience, and second, in what temporal window(s) can subsequent experiences build upon this signaling to maximize the induction of LTM? These questions can be most directly explored in the analysis of LTM induction across just two spaced experiences. During two-trial training the contribution of each trial to the induction of memory can be easily understood: the initial experience (Trial 1) recruits signaling events that must interact with a subsequent experience (Trial 2) provided within a temporally bounded window to establish LTM.

In *Aplysia*, the simplest training interval for LTM for sensitization is defined in part through the temporally restricted re-

cruitment of MAPK by an initial experience. However, we do not expect that 45 min will be the optimal training interval for two-trial memory induction in other systems or that this interval will even be optimal for other forms of learning in *Aplysia*. Rather, the optimal interval may be determined by kinetics for the recruitment of MAPK following a wide range of training stimuli which will have their own unique temporal constraints. Moreover, there are likely additional two-trial training intervals that will be revealed through a reexamination of many of the currently employed multiple trial protocols. As a striking example, Parsons and Davis (2012) recently re-examined multiple trial memory formation for fear-potentiated startle in rats and identified a remarkable two-trial training window that ranged from 1 h to several days. Finally, studies in humans have made clear that the determination of optimal training intervals for LTM formation depends heavily upon the retention interval examined (Donovan and Radosevich, 1999; Cepeda et al., 2006; Cepeda et al., 2008). Thus several factors influence the benefit of specific training intervals. Continued efforts to understand the molecular mechanisms which underlie the sensitivity to this wide range of training patterns should provide new insights into this fundamental feature of memory formation.

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