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Journal

Learning & Memory, 21(4)

ISSN

1072-0502

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Publication Date

2014-04-01

DOI

10.1101/lm.033894.113

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Activity-dependent inhibitory gating in molecular signaling cascades induces a novel form of intermediate-term synaptic facilitation in *Aplysia californica*

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Mechanistically distinct forms of long-lasting plasticity and memory can be induced by a variety of different training patterns. Although several studies have identified distinct molecular pathways that are engaged during these different training patterns, relatively little work has explored potential interactions between pathways when they are simultaneously engaged in the same neurons and circuits during memory formation. *Aplysia californica* exhibits two forms of intermediate-term synaptic facilitation (ITF) in response to two different training patterns: (1) repeated trial (RT) ITF (induced by repeated tail nerve shocks [TNSs] or repeated serotonin [5HT] application) and (2) activity-dependent (AD) ITF (induced by sensory neuron activation paired with a single TNS or 5HT pulse). RT-ITF requires PKA activation and de novo protein synthesis, while AD-ITF requires PKC activation and has no requirement for protein synthesis. Here, we explored how these distinct molecular pathways underlying ITF interact when both training patterns occur in temporal register (an “Interactive” training pattern). We found that (1) RT, AD, and Interactive training all induce ITF; (2) Interactive ITF requires PKC activity but not de novo protein synthesis; and (3), surprisingly, Interactive training blocks persistent PKA activity 1 h after training, and this block is PKC-independent. These data support the hypothesis that sensory neuron activity coincident with the last RT training trial is sufficient to convert the molecular signaling already established by RT training into an AD-like molecular phenotype.

Long-lasting memory and synaptic plasticity can be induced by a variety of different training patterns. Some tasks require repeated training trials (Ebbinghaus 1885/1915; Fanselow and Tighe 1988; Sutton et al. 2002; Philips et al. 2007, 2013), whereas other tasks require only a single training trial (Sutton et al. 2004; Izquierdo et al. 2006; Diamond et al. 2007; Shobe et al. 2009). Many studies have explored the molecular pathways that are engaged by different training patterns to produce plasticity and memory. Relatively little work has been done examining the potential interactions among these pathways when they are simultaneously engaged in the same cells and circuits during memory formation.

Aplysia californica is a useful model system for examining the molecular mechanisms underlying learning and memory (Hawkins et al. 2006). Intermediate-term memory (ITM) and its synaptic correlate, intermediate-term facilitation (ITF), are expressed 35–80 min after training, and can be induced by two different training patterns: repeated trial (RT) training and activity-dependent (AD) training (Sutton and Carew 2000, 2002; Stough et al. 2006). RT-ITM and RT-ITF are mediated by serotonin (5HT) neuromodulation at the tail sensory neuron (SN) and SN–motor neuron (MN) synapse, and are induced following spaced training trials (tail shocks; Sutton and Carew 2000; Marinesco and Carew 2002; Marinesco et al. 2006). AD-ITM and AD-ITF are

also mediated by 5HT neuromodulation, but in conjunction with SN activity, and require only a single training trial (Sutton and Carew 2000; Shobe et al. 2009). Both RT and AD forms of plasticity require MAPK activity for their induction and can last on average 90 min (Sutton and Carew 2000; Sharma et al. 2003). Interestingly, RT-ITM/ITF require de novo protein translation and PKA activity (Ghirardi et al. 1995; Sutton and Carew 2000; Ye et al. 2012), whereas AD-ITM/ITF require PKC activity and have no requirement for protein synthesis (Sutton and Carew 2000; Shobe et al. 2009).

The differential mechanistic requirements for the RT and AD forms of intermediate-term plasticity have provided a unique opportunity to explore how these pathways interact when both training patterns are engaged within the same neurons. To address this question, we developed an “Interactive” training pattern, in which typical spaced RT training trials are employed, but SN activity is made coincident with the last RT training trial. Thus the last RT trial is formally identical to AD-ITF training. We found that (1) RT, AD, and Interactive training all induce ITF; (2) Interactive ITF requires PKC activity but not de novo protein synthesis; and (3) Interactive training blocks persistent PKA activity 1 h after

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Article is online at <http://www.learnmem.org/cgi/doi/10.1101/lm.033894.113>.

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training in a PKC-independent manner. These data show that AD signaling within SNs coincident with the last RT training trial is sufficient to convert the RT-established molecular signaling into a single-trial, AD-like molecular phenotype. Thus, when both training patterns are simultaneously employed, one pattern (AD) overrides the other (RT), and does so by exerting an inhibitory influence in the molecular cascade activated by the overridden pattern.

Results

Coincident SN activity on the last RT training trial attenuates the expression of RT-ITF

To determine whether SN activity coincident with the final RT training trial affects expression and/or magnitude of ITF typically observed after RT training, we developed an Interactive stimulation pattern which consisted of five RT training trials (five spaced tail nerve shock [TNS] training trials, 30 V, 40 Hz, 2 sec, inter-trial interval [ITI] = 15 min) combined with a train of activity in the SN overlapping with the fifth RT TNS (Fig. 1A). We then characterized the ITF resulting from AD, RT, and Interactive training patterns. We found that all training patterns induced significant ITF 35–80 min after training relative to pretraining baseline EPSP levels (Wilcoxon matched-pairs signed rank: RT, $W = 21$, $n = 6$, $P = 0.031$; AD, $W = 28$, $n = 7$, $P = 0.016$; Interactive, $W = 26$, $n = 7$, $P = 0.031$) (Fig. 1B,C). The modestly decreased expression of

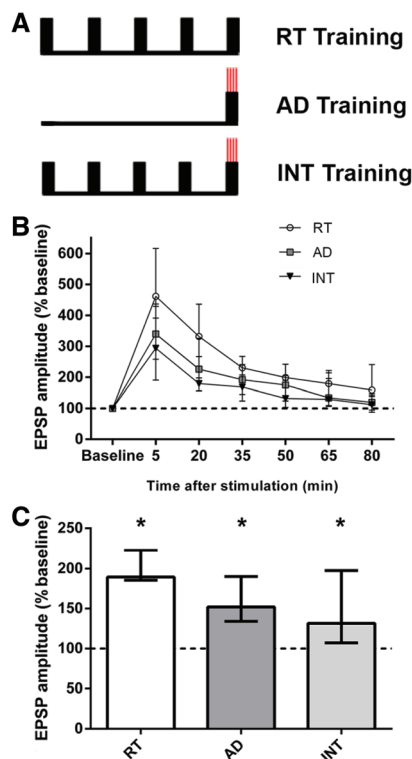


Figure 1. SN activity during Interactive training attenuates RT-ITF. (A) Schematic representation of training paradigms. RT training consists of five TNSs (black vertical bars, ITI = 15 min). AD training consists of a single TNS accompanied by sensory neuron (SN) activity (indicated by red hash marks). Interactive (INT) training is RT training with the last TNS combined with SN activity. (B) Time course of ITF expression (median EPSP amplitude \pm IQR). (C) Data from B showing significant ITF induction at 35- to 80-min post-training relative to pretraining baseline EPSPs by Wilcoxon matched-pairs signed rank test ($[*] P < 0.05$).

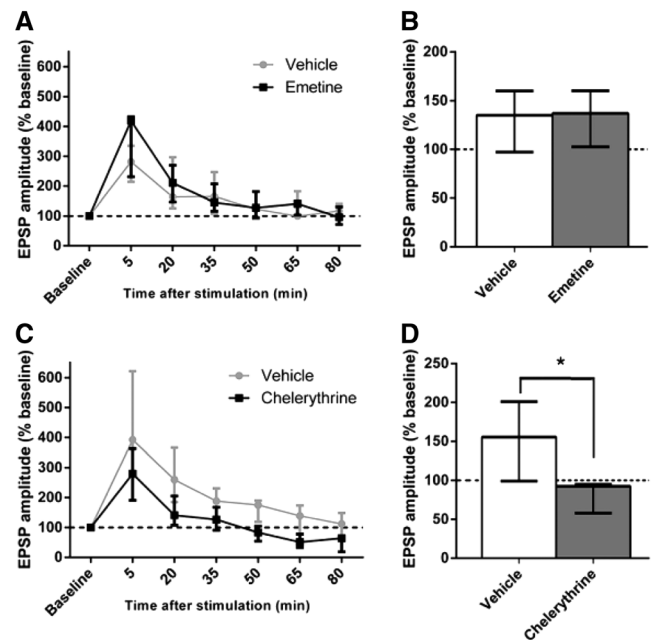


Figure 2. Interactive ITF requires PKC activity but not new protein synthesis. (A,B) Ganglia were incubated with the protein synthesis inhibitor emetine (100 μ M) or vehicle (ASW) for 30 min prior to training and throughout testing. (A) Time course of ITF expression (median EPSP amplitude \pm IQR). (B) Pooled data from A showing median EPSP amplitude (\pm IQR) for ITF (35–80 min). (C,D) Ganglia were incubated with the PKC inhibitor chelerythrine (10 μ M) or vehicle (0.05% DMSO) for 30 min prior to training and throughout testing. (C) Time course of ITF expression (median EPSP amplitude \pm IQR). (D) Pooled data from C showing median EPSP amplitude (\pm IQR) for the ITF temporal domain. Between group (vehicle vs. drug) analyses were performed by Mann–Whitney U tests ($[*] P < 0.05$).

Interactive ITF relative to RT-ITF raised the intriguing possibility that SN activity on the last RT training trial may attenuate RT-ITF. These results lead to the hypothesis that AD training can override RT-ITF when both forms of plasticity are induced concurrently.

Interactive ITF does not require protein synthesis

Since the expression data raise the possibility that AD training overrides RT signaling, we next set out to establish the molecular induction requirements for Interactive ITF. New protein synthesis is required during the induction of RT-ITF, whereas AD-ITF is independent of translation (Ghirardi et al. 1995; Sutton and Carew 2000). We next asked whether Interactive ITF, which is phenotypically similar to AD-ITF, is also mechanistically similar to AD-ITF, and therefore translation-independent. To examine this question, ganglia were incubated with the protein synthesis inhibitor emetine (100 μ M) or vehicle (ASW) 30 min prior to training and throughout the experiment. Although this treatment is sufficient to disrupt RT-ITF and memory in *Aplysia* (Sutton and Carew 2000), we found that emetine had no effect on Interactive ITF relative to vehicle control groups (Mann–Whitney U , $U = 7$, $n = 4$, $P = 0.829$) (Fig. 2A,B). Thus, a brief bout of SN activity at the end of RT training is sufficient to convert translation-dependent RT-ITF into a form of plasticity that no longer requires translation.

Interactive ITF requires PKC activity

AD-ITF is also uniquely dependent upon PKC, whereas RT-ITF is independent of PKC (Sutton and Carew 2000). Thus, we explored

whether Interactive ITF also shares this molecular requirement of AD-ITF. To test this notion, ganglia were incubated with the PKC inhibitor chelerythrine (10 μ M) or vehicle (0.05% dimethyl sulfoxide [DMSO]) 30 min prior to training and throughout the experiment. We found that blocking PKC completely abolished Interactive ITF relative to vehicle control groups (Mann–Whitney U , $U = 4$, $n = 6$, $P = 0.02$) (Fig. 2C,D), indicative of an AD-ITF-like plasticity. Taken together, these data are consistent with the idea that Interactive ITF and AD-ITF are phenotypically and mechanistically similar, if not identical.

Analog treatment for neuronal activity coincident with the last RT trial inhibits persistent PKA activity in a PKC-independent manner

The data thus far have provided evidence of an inhibitory interaction between SN activity and RT-ITF, and imply that SN activity may be sufficient to convert RT-ITF to AD-ITF. How might SN activity override the signaling recruited by RT training? One possibility is that AD training actively inhibits the development of RT plasticity. To test this possibility we examined a critical molecular step in the induction of RT-ITF. RT training induces persistent PKA activity 1 h after training, which is required for the expression of RT-ITF/ITM (Muller and Carew 1998; Sutton and Carew 2000; Sutton et al. 2001; Ye et al. 2012). Thus, we examined PKA activity levels in SNs 1 h after Analog Interactive ITF training, which increases Ca^{2+} levels in the ganglia due to an elevated KCl level which is used as a proxy for SN activity (Fig. 3A₁; see Methods and Materials, Shobe et al. 2009). Confirming previous observations (Sutton and Carew 2000), RT training induced significant PKA activation in SNs relative to within-animal control ganglia (Wilcoxin matched-pairs, $W = 55$, $n = 10$, $P = 0.002$); however, PKA activation was not observed following Analog Interactive training (relative to within-animal control ganglia; Wilcoxin matched-pairs, $W = -13$, $n = 10$, $P = 0.557$) (Fig. 3A₂). Furthermore, there was a significant difference between PKA activation in ganglia administered RT training and Analog Interactive training (Mann–Whitney U , $U = 21$, $n = 10$, $P = 0.03$) (Fig. 3A₂). These data are consistent with the hypothesis that increases in intracellular Ca^{2+} , a result of SN activity, actively inhibit RT-training induced PKA activation. Since Interactive ITF requires PKC activation, in a final experiment we asked whether this PKC signaling (Fig. 2D) might inhibit persistent PKA activation during Analog Interactive ITF induction. Intriguingly, treatment with the PKC inhibitor chelerythrine did not rescue 1-h PKA activity relative to vehicle control groups (Fig. 3B), indicating that Ca^{2+} increases coincident with the last RT trial induce inhibition of PKA activation, but do not do so through PKC signaling.

Discussion

In *Aplysia*, RT training induces ITM and ITF that require PKA and protein synthesis. In contrast, AD training induces a phenotypically similar ITM and ITF that are mechanistically distinct. This form of plasticity is PKC-dependent and translation- and PKA-independent (Sutton and Carew 2000; Shobe et al. 2009; Ye et al. 2012). In the present study, we examined the phenotype and mechanistic profile of plasticity that is induced when these two signaling cascades are engaged concurrently by combining SN activity with the last of five RT training trials to create an Interactive training pattern. A model for this type of interaction is shown in Figure 4.

Although RT and AD training both evoke 5HT-mediated increases in cAMP, the activation of G-proteins, and MAPK activation (Sutton and Carew 2000; Marinesco and Carew 2002;

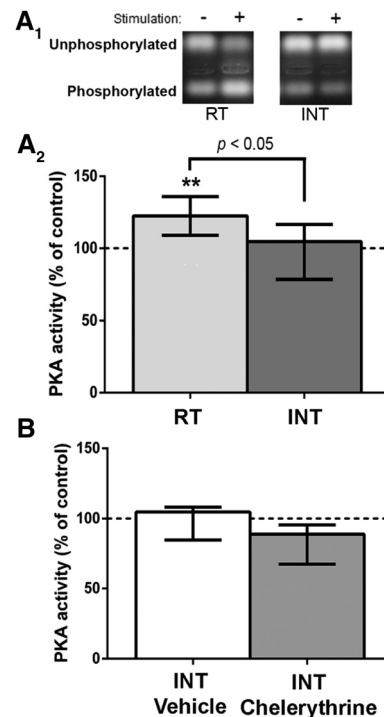


Figure 3. SN activity disrupts persistent PKA activation. PKA activity is expressed as the ratio of phosphorylated peptide in the experimental condition relative to controls. (A) RT training induces significant PKA activity 1 h after training relative to within-animal control ganglia levels, which is blocked by Interactive (INT) training. (A₁) Representative images from PKA assay showing differential migration of phosphorylated PKA substrate. (A₂) Data are plotted as median level of PKA activity (\pm IQR). Within-group analyses were performed with Wilcoxin matched-pairs signed rank test and between-group analyses were performed with Mann–Whitney U tests (** $P < 0.01$). (B) PKC inhibition by chelerythrine does not prevent the disruption of PKA activity during Analog Interactive training.

Marinesco et al. 2006; Ye et al. 2008, 2012; Shobe et al. 2009), SN activity is unique to AD training. SN activity recruits intracellular increases in Ca^{2+} and PKC activation (Sutton et al. 2004; Shobe et al. 2009), whereas RT training, in the absence of SN activity, leads to PKA activation (Ghirardi et al. 1995; Sutton and Carew 2000; Ye et al. 2012). Our results show that when SN activity is induced (or intracellular Ca^{2+} is increased) coincident with the last RT training trial, the typically established RT-ITF is now transformed into Interactive ITF, which phenotypically and mechanistically resembles AD-ITF. Specifically, Interactive ITF requires PKC activity but not protein synthesis. Moreover, increased intracellular Ca^{2+} on the last RT trial completely abolishes persistent PKA activity, a signature of RT-induced plasticity. Interestingly, despite the PKC requirement for induction of Interactive ITF, blocking PKC does not rescue persistent PKA activity, indicating that SN activity engages additional cellular and molecular changes that transform the established “memory” trace into a completely mechanistically distinct trace.

It is important to note that, although persistent PKA activity is inhibited by Analog Interactive training, PKA activity may still be required to establish the molecular environment permissive for the induction of Interactive ITF. Furthermore, our data indicate that Ca^{2+} increases are an integral component of SN activity contributing to the molecular signature of Interactive ITF, but these observations do not rule out the contribution of additional molecular cascades induced by SN activity that are not captured by increasing intracellular Ca^{2+} alone.

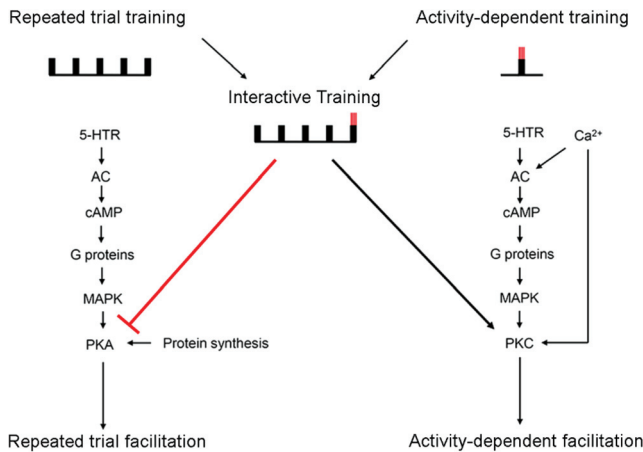


Figure 4. Model for activity-dependent inhibitory gating in Interactive ITF. Both RT and AD training induce a neuromodulatory response mediated by TNS-induced release of serotonin (5HT), which causes an increase in cAMP levels and stimulation of G-proteins upstream of MAPK activation. RT training exclusively recruits 5HT release in the absence of SN activity, which induces a protein-synthesis dependent PKA activation that underlies the expression of RT-ITF. AD training recruits 5HT release coupled with SN activity and Ca²⁺ influx, which induces PKC activation that ultimately mediates the expression of AD-ITF. Interactive training, which pairs the last RT training trial with SN activity (equivalent to an AD training trial), results in PKC-dependent and protein synthesis-independent ITF, despite having already established the molecular signaling important for RT-ITF. In addition, SN activity on the last RT training trial results in the PKC-independent inhibition of persistent PKA activation.

Mechanistically distinct forms of behavioral and synaptic plasticity in *Aplysia*

It has long been appreciated that memory can be induced by a variety of training patterns, often via canonical signaling cascades such as MAPK, PKA, and PKC. However, it is now becoming clear that apparently similar forms of memory or synaptic plasticity are actually mediated by very different molecular mechanisms. For example, Farah et al. (2009) demonstrated that PKC is differentially engaged by five spaced 5HT trials vs. a 90-min 5HT application, both of which are permissive for inducing long-lasting plasticity in *Aplysia* (Zhang et al. 1997; Mauelshagen et al. 1998). During spaced training, PKC is recruited to the plasma membrane after one training trial, but translocation becomes desensitized during subsequent training trials. Conversely, during massed training, PKC translocates to the plasma membrane and remains there with significantly less desensitization throughout training. Surprisingly, inhibiting PKA activity during spaced training rescues the desensitization of PKC translocation, allowing PKC to translocate after later training trials (Farah et al. 2009). These data provide compelling evidence for inhibitory crosstalk between the PKA and PKC in response to specific types of training patterns and are consistent with our observation that Interactive training recruits PKC and inhibits PKA activation. Similarly, Lorenzetti et al. (2006) found that classical and operant conditioning differentially regulate neuronal intrinsic excitability, suggesting that both molecular and cellular outcomes can be sensitive to the pattern of training.

Another example of pattern specific molecular plasticity was shown by Ye et al. (2008), who found that the ratio of small G-proteins Ras and Rap in the SNs of *Aplysia* (ApRas and ApRap) is regulated differently by different training patterns. Specifically, RT training induces plasticity in which ApRas activation exceeds that of ApRap, while AD training induces a reversed pattern in which ApRap activation exceeds that of ApRas. Importantly, ma-

nipulation of these ratios by overexpression of ApRas or ApRap altered MAPK activation in response to training (Ye et al. 2008). Specifically, MAPK activation by AD training was blocked when ApRas > ApRap, while the reverse was true for RT training (Ye et al. 2008), suggesting that imposition of a second, competing training paradigm could dampen or inhibit the molecular signaling cascades established by an initial training paradigm. These data raise the intriguing possibility that the molecular identity of a memory trace conferred by a unique training pattern may be established as early in molecular processing as the level of G-protein activation.

Finally, examining the SN-MN synapse in culture, Hu and colleagues (2006, 2007) have shown that different training patterns engage different signaling cascades to induce the release of the neuropeptide sensorin, which is required for LTF in *Aplysia*. RT training induces PKA- and PI3K-dependent synthesis and release of the neuropeptide sensorin (Hu et al. 2006), while AD training induces PKC-dependent synthesis of sensorin (Hu et al. 2007). Interestingly, the latter effect also requires the presence of the motor neuron (Hu et al. 2007), indicating that RT and AD training paradigms could be differentially engaging pre- and post-synaptic mechanisms.

Collectively, the studies discussed above indicate that a number of upstream molecular events (e.g., G-protein activation and postsynaptic engagement) may be contributing to the transformation of RT-ITF into AD-like ITF by Interactive training that we report in the present paper. It will now be of considerable interest to determine the specific mechanisms that mediate the transformation in underlying molecular architecture of plasticity induced by sensory neuron activity during Interactive training.

Alteration of a memory trace

Our results show that SN activity on a final RT training trial is sufficient to transform a PKA- and translation-dependent ITF to a PKC-dependent and translation-independent ITF. This was surprising given the fact that only four spaced RT training trials are sufficient to produce RT-ITF (Sutton et al. 2002), and indicates that coincident SN activity not only induces AD-like plasticity, but interacts with the signaling cascades already set in motion by previous RT trials (by inhibiting PKA activation). As depicted in our working model (Fig. 4), SN activity recruits both intracellular Ca²⁺ and PKC activation. Interestingly, PKC activity is not required for the inhibition of persistent PKA activity known to support RT-ITF. These considerations raise the question: how might Ca²⁺-dependent molecular and cellular events that differentiate AD and RT training ultimately inhibit PKA activity during Interactive training? PKA activity is dependent upon increases in cAMP, which cause dissociation of the regulatory subunits (RI and RII) from the catalytic subunits (Abel and Nguyen 2008). The catalytic subunits are then free to phosphorylate their substrates. A block of PKA activation could result from a number of different mechanisms, including a change in the relative levels of free regulatory or catalytic subunits that would favor reassociation, thereby diminishing the level of PKA activity. It was previously established that PKA RI subunits are targeted for proteosomal degradation by induction of the immediate early gene, *ApUch*, during the induction of LTF in *Aplysia* (Bergold et al. 1992; Hegde et al. 1997).

Interestingly, PKA RII subunits are transcriptionally and translationally up-regulated and targeted to the plasma membrane during LTF, where they are tethered to the plasma membrane by A kinase anchoring-proteins (AKAPs; Liu et al. 2004). AKAP-RII subcellular localization can be changed by Ca²⁺ and PKC, notably from the plasma membrane to the cytoplasm (Yan et al. 2009; Schott and Grove 2013). This redistribution, in

principle, could both decrease the likelihood of cAMP stimulation of PKA as well as alter the rate of regulatory and catalytic subunit reassociation by physically changing the location of regulatory subunits. Intriguingly, AKAPs can bind both PKA and calcineurin near AMPA receptors, thereby increasing bidirectional modulation of receptor phosphorylation (Dell'Acqua et al. 2006) and brokering dynamic changes in synaptic plasticity.

Molecular identity of Interactive ITF

Our data indicate that RT-ITF can obtain a different mechanistic signature by virtue of SN activity coincident with the final RT training trial. This new Interactive ITF shares the molecular hallmarks of AD-ITF, but whether Interactive ITF is, in fact, the same as AD-ITF, or whether ITF has distinct characteristics from both RT- and AD-ITF remains an important question. It may be the case that an AD-like training trial supersedes RT training, even if RT signaling cascades are already activated. Alternatively, the Interactive plasticity we have identified may provide a unique opportunity to study the cellular and molecular mechanisms of a novel form of metaplasticity, which in turn could provide a mechanism for the integration of different learning experiences into a single neural network. Interactive plasticity, as well as additional forms of metaplasticity in other model systems (for review, see Schmidt et al. 2013), may provide a general mechanism for the integration of novel experiences into a single neural network using highly conserved and interactive molecular mechanisms.

Materials and Methods

Synaptic facilitation in intact CNS

Wild-caught *A. californica* (150–250 g, obtained from Marinus Scientific, Long Beach, CA or Santa Barbara Marine Bio., Santa Barbara, CA) were anesthetized with isotonic MgCl₂. Ganglia were prepared for electrophysiological studies of the tail SN–MN synapse as described in Sharma et al. (2006). At least two pretests followed any drug application to verify lack of baseline effects. Facilitation was induced using RT, AD, or Interactive (INT) stimulation. STF and ITF were examined by eliciting a single action potential in the SN every 15 min from 5 to 80 min after the end of stimulation and recording the resulting EPSP amplitudes in the MN.

RT, AD, and interactive training patterns

RT training consists of five spaced p9 tail nerve shocks (TNSs; 30 V, 40 Hz, 5-msec pulse duration, 2-sec train duration, inter-trial interval [ITI] = 15 min). AD training consists of a single TNS followed by four trains of suprathreshold current pulses in the SN (10 Hz, 4-msec pulse duration, 2-sec train duration) occurring 5, 15, 25, and 35-sec post-TNS to ensure SN activity is coincident with serotonin release stimulated by TNS (Marinesco and Carew 2002). Interactive training consists of four spaced TNS (ITI = 15 min) with the fifth spaced TNS followed immediately by four trains of SN activation in a manner analogous to that for AD stimulation.

Interactive Analog training for molecular analyses

For the molecular studies, we used a slightly modified Interactive training protocol. SN activation by current injection on the fifth RT trial was mimicked by incubation in a depolarizing high-KCl artificial seawater (ASW; 100 mM) for 5 min immediately following the fifth RT TNS, as described in Shobe et al. (2009). The high-KCl solution was subsequently washed out with ASW. One-hour post-training, ganglia were washed with cold (4°C) 1:1 ASW:MgCl₂ and the SN cluster was excised, homogenized, and placed on dry ice.

Kinase assays

To study PKA activity, SN lysates were incubated with a positively charged peptide substrate containing a consensus PKA phosphorylation site (Kemptide) for 30 min, as described in Ye et al. (2012). Gels were analyzed using ImageJ, and PKA activity was quantified by comparing the ratio of phosphorylated peptide to total peptide (normalized for total protein). Persistent PKA activation was quantified by comparing PKA activation in experimental vs. within-animal control SN clusters.

Statistical analyses

Because the data were nonnormally distributed, we used nonparametric statistics for all between-group and within-group comparisons. Data were considered significant if $P < 0.05$. Significant expression of ITF was assessed by comparing average pretreatment/training baseline EPSPs to the average EPSP during the 35- to 80-min post-training time window using a Wilcoxon matched-pairs signed rank test. To test whether ITF is significantly different between groups (as in the case of vehicle vs. drug treatment), a Mann–Whitney U test was used. Similarly, PKA activity was assessed by the level of phosphorylated peptide in the experimental group compared to within-animal control levels using a Wilcoxon matched-pairs signed rank test. Between-group (vehicle vs. drug) statistics were performed with a Mann–Whitney U test.

Acknowledgments

We thank Dr. Gary Philips for very helpful comments on an earlier draft. We also thank Alexandria Vasquez for help with experiments. This work was supported by NIMH R01MH041083 and R01MH094792 and NSF Grant IOB-0444762 to T.J.C.

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Received November 12, 2013; accepted in revised form January 31, 2014.