Rescue of Impaired Long-Term Facilitation at Sensorimotor Synapses of *Aplysia* following siRNA Knockdown of CREB1

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Memory impairment is often associated with disrupted regulation of gene induction. For example, deficits in cAMP response element-binding protein (CREB) binding protein (CBP; an essential cofactor for activation of transcription by CREB) impair long-term synaptic plasticity and memory. Previously, we showed that small interfering RNA (siRNA)-induced knockdown of CBP in individual sensory neurons significantly reduced levels of CBP and impaired 5-HT-induced long-term facilitation (LTF) in sensorimotor cocultures from *Aplysia*. Moreover, computational simulations of the biochemical cascades underlying LTF successfully predicted training protocols that restored LTF following CBP knockdown. We examined whether simulations could also predict a training protocol that restores LTF impaired by siRNA-induced knockdown of the transcription factor CREB1. Simulations based on a previously described model predicted rescue protocols that were specific to CREB1 knockdown. Empirical studies demonstrated that one of these rescue protocols partially restored impaired LTF. In addition, the effectiveness of the rescue protocol was enhanced by pretreatment with rolipram, a selective cAMP phosphodiesterase inhibitor. These results provide further evidence that computational methods can help rescue disruptions in signaling cascades underlying memory formation. Moreover, the study demonstrates that the effectiveness of computationally designed training protocols can be enhanced with complementary pharmacological approaches.

Introduction

Specific defects in biochemical pathways are correlated with impairments in human cognition, learning, and memory (Raymond and Tarpey, 2006; Roelfsema and Peters, 2007; Saura and Valero, 2011; Stornetta and Zhu, 2011; Wijetunge et al., 2013). Animal models of molecular deficits mimic these impairments and are critical to test the efficacy of potential treatments. For example, cAMP response element-binding protein (CREB) binding protein (CBP) deficits impair hippocampal long-term potentiation (LTP) and long-term memory (LTM) (Wood et al., 2005; Barrett et al., 2011), and CBP mutations are the most common cause of Rubinstein–Taybi syndrome (RTS). CBP mutant mice serve as models of RTS (Bourtchouladze et al., 2003; Alarcón et al., 2004; Korzus et al., 2004). In these mice, some memory impairment is rescued by treatment with rolipram, an inhibitor of cAMP phosphodiesterase 4 (PDE4). Rolipram also rescues cognitive deficits in mouse models of Alzheimer's disease and traumatic brain injury (Gong et al., 2004; Titus et al., 2013), and enhances LTP and hippocampal-dependent memory (Barad et al., 1998; Bourtchouladze et al., 2003). The facilitatory effects of rolipram are presumably due to an enhancement of protein ki-

nase A (PKA) activity, thereby increasing phosphorylation of CREB by PKA (MacKenzie and Houslay, 2000; Bourtchouladze et al., 2003).

The use of computational simulations to design optimal training protocols is an alternative, but not necessarily mutually exclusive, approach for the enhancement of memory. For example, a model that describes the dynamics of the cAMP/PKA and rapidly accelerated fibrosarcoma (Raf)/MEK/ERK signaling pathways, both of which are essential for LTF (Martin et al., 1997; Chain et al., 1999), predicted that specific, irregular spacing between trials would maximize the production of key downstream transcription factors and thereby enhance LTF and the formation of LTM (Zhang et al., 2012). These factors include the phosphorylated, activated forms of CREB1 (Bartsch et al., 1998) and CCAAT/enhancer binding protein (C/EBP) (Alberini et al., 1994). C/EBP, when phosphorylated by ERK, activates genes essential for LTF (Alberini et al., 1994; Yamamoto et al., 1999). ERK also phosphorylates the repressor CREB2, relieving repression of transcription (Bartsch et al., 1995).

In addition to enhancing normal memory, this computational strategy predicted a rescue protocol that restored a deficit in LTF associated with an *in vitro* model of RTS. Liu et al. (2013) used small interfering RNA (siRNA) to reduce the basal level of CBP and impair LTF at sensory neuron–motor neuron (SN–MN) synapses of *Aplysia*. The mathematical model of Zhang et al. (2012) was extended to include the cAMP/PKA and Raf/MEK/ERK pathways, as well as phosphorylation of CREB1, CREB2, and C/EBP. Simulations predicted training protocols that would compensate for the CBP deficit. These rescue protocols induced substantial temporal overlap of CREB1 activation and CREB2 inactivation by repeated, irregularly spaced stimuli. Subsequent

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empirical studies validated the strategy by confirming that one of these rescue protocols restored LTF.

In the present study, we examine whether this strategy can also be used to rescue a deficit in LTF produced by a different molecular lesion, knockdown of CREB1 induced by siRNA. In addition, we examine the effects of combining the traditional pharmacological approach with computationally designed rescue protocols.

Materials and Methods

Cell culture, siRNA injection, and electrophysiology. SN–MN cocultures from *Aplysia* were prepared as described previously (Zhang et al., 2012). A single SN and a single L7 MN were plated onto each dish to make SN–MN cocultures. Two to three days after plating, the SN was injected with siRNA (5 μ M) following established procedures (Liu et al., 2011a). Pools of CREB1–siRNA (Dharmacon Research) contained four different sequences of siRNA (Liu et al., 2011a). Scrambled siRNA (Dharmacon Research) was used as an injection control (Con-siRNA). After injection, SN–MN cocultures were allowed to grow for another 72 h at 18°C. Before pretesting the synaptic connection, the growth medium was replaced by a recording solution that consisted of 50% isotonic L15 and 50% artificial seawater [ASW; containing (in mM) 450 NaCl, 10 KCl, 11 CaCl₂, 29 MgCl₂, and 10 HEPES, pH 7.6; L15–ASW]. EPSPs were recorded from MNs with 10–20 M Ω sharp electrodes filled with 3 M potassium acetate. MNs were hyperpolarized to –90 mV to prevent action potentials. Sensory neurons were stimulated via a blunt patch electrode filled with L15–ASW. Cultures were excluded from further analysis if pretest EPSPs were <5 mV or >35 mV, which tended to generate action potentials after 5-HT treatment (Zhang et al., 2012). Following pretests, SN–MN cocultures were exposed to five 5 min pulses of 50 μ M 5-HT to induce LTF. Post-test EPSPs were recorded 24 h after 5-HT treatment. Experiments were done in a blind manner following published procedures (Liu et al., 2013). Data acquisition was performed using pClamp version 10 (Molecular Devices). All experiments were performed at room temperature (20–25°C).

Data analysis and statistics. LTF was quantified as the percentage increase of the post-test EPSP at 24 h after 5-HT treatment relative to the pretest EPSP. LTF measurements were excluded if values were outside 3 SDs from the mean. Two experiments were excluded based on this criterion. Parametric tests were used in all experiments because data passed the Shapiro–Wilk normality test. Statistical tests were performed using Sigmaplot 11 (Jandel Scientific). Differences between means were considered significant at $p < 0.05$.

Drugs. Stock solutions of 5-HT (5 mM; Sigma-Aldrich) were prepared fresh in L15–ASW on the day of the experiment. Rolipram (Sigma-Aldrich) was prepared as a 20 mM stock solution in DMSO and stored at –20°C. On the day of an experiment, an aliquot of the rolipram stock solution was diluted in L15–ASW to a final concentration of 0.2 μ M. For groups treated with rolipram (0.2 μ M), the drug was applied 30 min before 5-HT or vehicle (Veh) treatment and continued throughout the experiment.

Computational model. The model of Liu et al. (2013) was extended to include the actions of CREB1–siRNA and rolipram. The model describes activation of PKA and ERK, phosphorylation of CREB1 and CREB2, and synthesis and phosphorylation of C/EBP. The output of the model, predictive of the relative strength of LTF, was taken to be the peak level of phosphorylated C/EBP (pC/EBP), which is a point of convergence for several second-messenger and transcriptional pathways.

With two exceptions, the equations and parameter values of the model were as described previously [Zhang et al. (2012), their Eqs. 1–12; Liu et al. (2013), their Eqs. 1–7]. To simulate the effects of siRNA knockdown, CREB1 $[[CREB1]_{total}]$ in Eq. 1 of Liu et al. (2013) was reduced by 15%, which was equivalent to the average empirical siRNA-mediated knockdown (see Results). The reduction of $[[CREB1]_{total}]$ led to a decreased level of pC/EBP, in agreement with the empirical attenuation of LTF.

In *Aplysia*, rolipram inhibits an isoform of PDE4 (ApPDE4) (Park et al., 2005). To simulate the effect of rolipram on LTF, the equation describing the synthesis and degradation of cAMP was modified as follows:

$$d[cAMP]/dt = \lambda[5-HT]/([5-HT] + K_{5-HT}) - k_{b,cAMP}[PDE][cAMP].$$

In the equation, PDE represents ApPDE4. The initial concentration of PDE was set as 1 μ M. The effect of rolipram was represented by the reduction of PDE (see Fig. 2A).

The model was programmed in XPPAUT 5.41 (www.math.pitt.edu/~bard/xpp/xpp.html). Fourth-order Runge–Kutta integration was used, with a time step of 3 s. Source codes are available upon request. The model has been uploaded to the ModelDB database (<https://senselab.med.yale.edu/modeldb/default.asp>, model number 149162).

Results

Knockdown of CREB1 expression by siRNA blocked LTF

Previously, we showed that LTF was blocked by injection of CREB1–siRNA within 12 h after a standard protocol of 5-HT treatment (Liu et al., 2011a). Here, “standard” protocol refers to five 5 min pulses of 50 μ M 5-HT, regularly spaced with an inter-stimulus interval (ISI) of 20 min (Montarolo et al., 1986). The siRNA specifically blocked 5-HT-induced increases in CREB1, but basal levels of CREB1 were not affected at 48 h after siRNA injection. At 72 h after injection, however, CREB1–siRNA significantly reduced basal levels of CREB1 by $15 \pm 4\%$ (mean $\pm$ SEM) compared to the control siRNA injections (Liu et al., 2011a). To test whether this reduction of basal CREB1 at 72 h after injection would impair LTF, empirical studies were performed in four groups of SN–MN cocultures (Fig. 1). In Groups 1 and 3, SNs were injected with control (scrambled) siRNA and treated with either the standard (ST) protocol of 5-HT (50 μ M) or with equivalent applications of vehicle (Fig. 1A, ST + Con-siRNA, black traces; Veh + Con-siRNA, blue traces). In Groups 2 and 4, SNs were injected with CREB1–siRNA and treated with either the standard protocol or with vehicle (Fig. 1A, ST + CREB1–siRNA, red traces; Veh + CREB1–siRNA, yellow traces). LTF was evaluated 24 h after application of 5-HT. In the 5-HT treatment groups, LTF was $184 \pm 18\%$ of the pretest EPSP with injection of Con-siRNA (ST + Con-siRNA, $n = 6$), whereas LTF was reduced to $121 \pm 10\%$ of pretest by injection of CREB1 siRNA (ST + CREB1–siRNA, $n = 7$). Vehicle treatment with injection of either Con-siRNA or CREB1 siRNA did not produce LTF (Veh + Con-siRNA, $102 \pm 6\%$, $n = 4$; Veh + CREB1–siRNA, $86 \pm 9\%$, $n = 5$). A one-way ANOVA indicated significant overall differences among treatment groups (Fig. 1B; $F_{(3,18)} = 11.82$, $p < 0.001$). Injection of CREB1 siRNA significantly impaired LTF. *Post hoc* pairwise comparison [Student–Newman–Keuls (SNK) method] indicated that the ST + Con-siRNA group was significantly different from the other three groups (Fig. 1B; ST + Con-siRNA vs ST + CREB1–siRNA, $q_2 = 5.44$, $p < 0.001$; ST + Con-siRNA vs Veh + CREB1–siRNA, $q_4 = 7.77$, $p < 0.001$; ST + Con-siRNA vs Veh + Con-siRNA, $q_3 = 6.14$, $p < 0.001$). Moreover, no significant differences were found among the ST + CREB1–siRNA, Veh + Con-siRNA, and Veh + CREB1–siRNA groups (Fig. 1B; ST + CREB1–siRNA vs Veh + CREB1–siRNA, $q_3 = 2.86$, $p = 0.14$; ST + CREB1–siRNA vs Veh + Con-siRNA, $q_2 = 1.49$, $p = 0.31$; Veh + Con-siRNA vs Veh + CREB1–siRNA, $q_2 = 1.11$, $p = 0.44$).

Prediction of a stimulus protocol that rescues LTF

Induction and phosphorylation of C/EBP represents the effect of convergence of the PKA signaling pathway, which enhances CREB1 phosphorylation, and the ERK signaling pathway, which inactivates CREB2 and phosphorylates C/EBP (Fig. 2A). CREB1 activation, CREB2 inactivation, and C/EBP phosphorylation are all required for LTF (Alberini et al., 1994; Bartsch et al., 1995, 1998; Yamamoto et al., 1999; Guan et al., 2003). Therefore, the increase in pC/EBP was considered a correlate of the relative extent of LTF and was used as the output of the mathematical

Figure 1. LTF is impaired by siRNA knockdown of CREB1. **A**, Representative traces of EPSPs immediately before (pretest) and 24 h after (post-test) the standard 5-HT protocol. SNs were injected with either CREB1 siRNA or Con-siRNA 72 h before the pretest. Effects of knockdown of CREB1 on LTF were examined in four treatment conditions: a standard protocol of 5-HT treatment in the presence of either (1) Con-siRNA (black trace) or (2) CREB1 siRNA (red trace) and a standard protocol of vehicle treatment in the presence of either (3) Con-siRNA (blue trace) or (4) CREB1 siRNA (yellow trace). In this and subsequent illustrations, the amplitude of pretest EPSPs is indicated by dashed lines. **B**, Summary data for LTF after the standard treatment. In this and subsequent illustrations, LTF was quantified by the percentage increase of the post-test EPSP relative to the pretest EPSP (% Pretest), and error bars represent the mean $\pm$ SEM. A one-way ANOVA indicated no significant differences in the amplitude of pretest EPSPs among the four groups ($F_{(3,18)} = 0.209$, $p = 0.89$). For this and subsequent illustrations, asterisks indicate the statistical results from *post hoc* pairwise comparisons. $^{**}p < 0.01$.

model (Liu et al., 2013). To simulate the magnitude of empirical reduction of CREB1 by siRNA, the model parameter $[CREB1]_{total}$ was reduced to 85% of the value used previously (Liu et al., 2013). With this reduction, simulations of the standard 5-HT protocol exhibited a decrease of peak phosphorylated C/EBP (pC/EBP) (Fig. 2B1,B2). The percentage decrease of simulated peak pC/EBP (from $\sim 0.08 \mu M$ with ST + Con-siRNA to $\sim 0.06 \mu M$ with ST + CREB1-siRNA; $\sim 30\%$ decrease) was comparable to the empirical impairment of LTF in Figure 1B (from 184% with ST + Con-siRNA to 121% with ST + CREB1-siRNA; 34% decrease). We next attempted to predict a rescue protocol that would compensate for reduced $[CREB1]$ and restore the simulated peak pC/EBP to the peak level produced by the standard protocol with normal $[CREB1]$. After simulation of 10^4 candidate protocols [performed as described by Liu et al. (2013)], 336 protocols were found to restore the simulated peak level of pC/EBP. Among these 336 potential rescue protocols, one was arbitrarily selected for empirical analysis (Fig. 2B3). This protocol had ISIs of 10, 15, 20, and 10 min, and is henceforth termed the “rescue protocol” (Res; Fig. 2B3). In simulations, the rescue protocol restored the peak level of pC/EBP above the peak level produced by the standard protocol without CREB1 knockdown (Fig. 2B1,B3; Res + CREB1-siRNA, $\sim 0.11 \mu M$ vs ST + Con-siRNA, $\sim 0.08 \mu M$). The peak level of pC/EBP simulated by the rescue protocol in the absence of CREB1 siRNA was $0.15 \mu M$, $\sim 35\%$ higher than the level in the presence of CREB1 siRNA. In the presence of CREB1 siRNA, the simulated peak level of pCREB1 was reduced by $\sim 18\%$ in both the standard and rescue protocols.

Because degradation of cAMP is an important step that limits the effectiveness of the signaling cascade, we hypothesized that the efficacy of the rescue protocol could be further enhanced by treatment with rolipram. As a first step, we simulated the effects of decreased PDE activity (ranging from 0 to 50%) on the peak level of pC/EBP in the CREB1 knockdown model with the standard protocol (Fig. 2C, blue). Decreases in PDE activity by more than $\sim 30\%$ restored the simulated peak pC/EBP to or above the level observed without CREB1 knockdown (Fig. 2C, arrow 2). We next simulated the effects of decreases in PDE paired with the rescue protocol (Fig. 2C, red). Decreased PDE further enhanced the peak level of pC/EBP induced by the rescue protocol. In addition, modest synergistic effects were observed when rolipram was included with

Figure 3. Dynamics of key signaling molecules during induction of LTF. **A1–E**, Black traces illustrate the responses of the model to the standard protocol, whereas red traces illustrate the responses to the rescue protocol. **A1, A2**, Time courses of PKA activity (the concentration of free catalytic subunit, PKA_C). **B**, Time courses of active ERK activity (pERK). **C**, Time courses of active CREB1 (pCREB1). **D**, Time courses of inactive CREB2 (pCREB2). **E**, Time courses of active C/EBP (pC/EBP). The black solid and black dashed time courses in **C** and **E** are for the standard protocol without and with CREB1 knockdown, respectively. The solid red traces in **C** and **E** are the time courses of the rescue protocol with CREB1 knockdown. **F**, 5-HT pulses and ISIs for the standard protocol (black) and rescue protocol (red). The standard protocol consisted of uniform ISIs of 20 min, whereas the rescue protocol consisted of nonuniform ISIs of 10, 15, 20, and 10 min.

Con-siRNA, $n = 12$), whereas LTF was reduced to $108 \pm 6\%$ of pretest by injection of CREB1 siRNA (ST + CREB1-siRNA, $n = 9$). The rescue protocol partially restored LTF to $133 \pm 7\%$ of pretest (Res + CREB1-siRNA, $n = 11$). A one-way ANOVA indicated significant overall differences among treatment groups (Fig. 4B; $F_{(2,29)} = 9.64$, $p < 0.001$). *Post hoc* pairwise comparison (SNK) indicated that LTF in the ST + CREB1-siRNA group was significantly less than in the ST + Con-siRNA group, confirming the effectiveness of the knockdown (Fig. 4B; ST + Con-siRNA vs ST + CREB1-siRNA, $q_3 = 6.2$, $p < 0.001$). In addition, LTF in the Res + CREB1-siRNA group was significantly greater than in the ST + CREB1-siRNA group, but also significantly smaller than in the ST + Con-siRNA group, indicating partial rescue of LTF (Fig. 4B; Res + CREB1-siRNA vs ST + CREB1-siRNA, $q_2 = 3.16$, $p < 0.05$; ST + Con-siRNA vs Res + CREB1-siRNA, $q_2 = 3.14$, $p < 0.05$).

Effectiveness of the rescue protocol was further enhanced by rolipram

The model predicted that rolipram would improve the effectiveness of the rescue protocol by increasing cAMP levels. First, we examined the effects of rolipram by itself on LTF. A concentration of 0.2μ M was selected because similar concentrations are effective in enhancing LTP (Barad et al., 1998; Titus et al., 2013). Four groups of SN–MN cocultures were examined (Fig. 5). Group 1 was treated with the standard protocol to confirm 5-HT-induced LTF (Fig. 5A, ST, black traces). Group 2 was a control treated with vehicle (Fig. 5A, Veh, red traces). For Groups 3 and 4, rolipram was applied 30 min before 5-HT or vehicle and was maintained in the medium until the end of 5-HT/vehicle treatment. LTF was evaluated 24 h later following application of 5-HT. In the standard 5-HT treatment groups, 5-HT alone yielded LTF of $146 \pm 9\%$ of pretest (ST, $n = 8$), and LTF was further enhanced to $165 \pm 8\%$ of pretest by rolipram (ST + Roli, $n = 10$). In the vehicle and rolipram alone treatment groups, little or no LTF was observed (Veh, $108 \pm 4\%$ of pretest, $n = 9$; Veh + Roli, $120 \pm 4\%$ of pretest, $n = 9$). A one-way ANOVA indicated significant overall differences among treatment groups (Fig. 5B; $F_{(3,32)} = 15.426$, $p < 0.001$). *Post hoc* pairwise comparison (SNK) indicated that LTF in the ST group was significantly greater than in the Veh and Veh + Roli groups, confirming that 5-HT induced LTF (Fig. 5B; ST vs Veh + Roli, $q_2 = 3.7$, $p < 0.05$; ST vs Veh, $q_3 = 5.4$, $p < 0.01$). Rolipram alone did not induce significant LTF as compared to the vehicle group (Veh + Roli vs Veh, $q_2 = 1.79$, $p = 0.22$). However, LTF in the ST + Roli group was significantly greater than in the other three groups (Fig. 5B; ST + Roli vs ST, $q_2 = 2.9$, $p < 0.05$; ST + Roli vs Veh + Roli, $q_3 = 6.95$, $p < 0.001$; ST + Roli vs Veh, $q_4 = 8.8$, $p < 0.001$). Therefore, rolipram enhanced 5-HT-induced LTF.

Next, we examined the effects of a combination of the rescue protocol with rolipram on the impaired LTF following CREB1 knockdown. Three groups of SN–MN cocultures were examined (Fig. 6). In all groups, SNs were injected with CREB1 siRNA. Group 1 was treated with the rescue protocol in the presence of 0.2μ M rolipram (Fig. 6A, Res + Roli + CREB1-siRNA, black traces). Group 2 was treated with the rescue protocol alone (Fig. 6A, Res + CREB1-siRNA, red traces). Group 3 was treated with 0.2μ M rolipram alone (Fig. 6A, Veh + Roli + CREB1-siRNA, blue traces). LTF was evaluated 24 h later following application of 5-HT. Pairing rolipram with the rescue protocol yielded LTF of $158 \pm 9\%$ of pretest (Res + Roli + CREB1-siRNA, $n = 11$), whereas the rescue protocol alone produced LTF of $132 \pm 8\%$ of pretest (Res + CREB1-siRNA, $n = 10$). Rolipram alone treatment in the presence of injection of CREB1 siRNA did not yield

significant LTF ($109 \pm 10\%$ of pretest, Veh + Roli + CREB1-siRNA, $n = 9$). A one-way ANOVA indicated significant overall differences among treatment groups (Fig. 6B; $p < 0.01$). LTF in the Res + Roli group was significantly greater than in the Res alone or Veh + Roli group (Fig. 6B; Res + Roli + CREB1-siRNA vs Veh + Roli + CREB1-siRNA, $q_3 = 5.52$, $p < 0.01$; Res + Roli + CREB1-siRNA vs Res + CREB1-siRNA, $q_2 = 3.1$, $p < 0.05$). These results illustrate that the effectiveness of the computational approach can be improved with a complementary pharmacological approach.

Discussion

The present study examined whether a computationally designed training protocol can be used to rescue a deficit in LTF produced by a knockdown of CREB1 induced by siRNA. In addition, it examined the effects of combining a rescue training protocol with a traditional pharmacological approach.

Rescue of impaired LTF

Extensive studies have demonstrated that CREB-related signaling plays an essential role in regulating LTM and long-term synaptic plasticity. In *Aplysia*, injection of *creb1* oligonucleotides, or of antibodies to CREB1, blocks 5-HT-induced LTF (Dash et al., 1990; Bartsch et al., 1998), as does injection of CREB1 siRNA (Liu et al., 2011a), a result that was confirmed in the present study (Fig. 1). In rodents, targeted knock-out of CREB isoforms disrupts long-term memory in several behavioral protocols, such as fear conditioning and spatial learning in the water maze (Bourtchuladze et al., 1994; Pittenger et al., 2002; Peters et al., 2009), whereas overexpression of CREB via viral transfection enhances fear conditioning (Josselyn et al., 2001; Restivo et al., 2009). In addition to CREB, CBP is also essential for LTM and long-term synaptic plasticity (Guan et al., 2002; Alarcón et al., 2004; Wood et al., 2005; Barrett et al., 2011). Previously, Liu et al. (2013) showed that the deficit in LTF produced by a reduction of CBP, a cellular model of RTS, could be rescued by a computationally designed training protocol. The present study demonstrates that same strategy can be used to rescue a deficit in LTF produced by a reduction of CREB1 levels.

The simulations of empirically validated rescue protocols for CREB knockdown (Fig. 3) and for CBP knockdown (Liu et al., 2013) suggest that successful establishment of LTM depends on sub-

Figure 4. Rescue protocol partially restored impaired LTF. **A**, Representative traces of EPSPs immediately before (pre-test) and 24 h after (post-test) either the standard or rescue protocols. SNs were injected with either CREB1 siRNA or Con-siRNA 72 h before the pretest. Three groups were examined: the standard protocol of 5-HT treatment in the presence of (1) Con-siRNA (black trace) and (2) CREB1 siRNA (red trace), and (3) the rescue protocol of 5-HT treatment in the presence of CREB1 siRNA (blue trace). The initial biphasic responses on the traces are stimulus artifacts. **B**, Summary data. Error bars represent the mean $\pm$ SEM. A one-way ANOVA indicated no significant differences in the amplitude of pretest EPSPs among the three groups ($F_{(2,29)} = 0.027$, $p = 0.97$). * $p < 0.05$; ** $p < 0.01$.

Figure 5. Rolipram enhanced LTF. **A**, Representative traces of EPSPs immediately before (pretest) and 24 h after (post-test) the standard protocol. In the absence of siRNA injection, effects of rolipram on LTF were examined in four treatment conditions: (1) the standard protocol of 5-HT treatment (black trace), (2) the standard protocol of vehicle treatment (red trace), (3) the standard protocol of vehicle treatment in the presence of rolipram (blue trace), (4) the standard protocol of 5-HT treatment in the presence of rolipram (yellow trace). **B**, Summary data. Error bars represent the mean $\pm$ SEM. A one-way ANOVA indicated that there were no significant differences in the amplitude of pretest EPSPs among the four groups ($F_{(3,32)} = 0.45$, $p = 0.71$). * $p < 0.05$; ** $p < 0.01$. n.s., Not statistically significant.

stantial overlap between mutually reinforcing elements of the PKA and ERK signaling pathways. For *Aplysia* and mammals, these events include PKA-mediated phosphorylation and activation of CREB/CREB1 and C/EBP, reinforced in *Aplysia* by ERK-mediated inactivation of CREB2. The strategy we developed suggests that modeling can successfully predict spaced training

protocols—specific, irregularly spaced ISI patterns—that optimize such overlap and enhance learning and LTM.

The rescue protocol for CREB1 knock-down, with ISIs of 10, 15, 20, and 10 min, is very similar to the previous rescue protocol for CBP knockdown (Liu et al., 2013), which had ISIs of 10, 10, 20, and 10 min. In simulations of both rescue protocols, the early short ISIs (relative to the standard protocol ISIs of 20 min) yield a more rapid activation of ERK, and therefore a more rapid phosphorylation of CREB2, relieving repression of *c/ebp* transcription. In both protocols, 5-HT pulses 4 and 5 are separated from pulses 1–3 by a longer ISI, which acts to create a second, distinct peak of CREB1 phosphorylation, increasing the overlap between CREB1 phosphorylation and CREB2 phosphorylation (Fig. 3). This increase also contributes to *c/ebp* induction. Indeed, of the 10,000 protocols that were simulated, 336 protocols restored the peak of phosphorylated C/EBP up to or above its normal value. One of these protocols was the CBP rescue protocol in Liu et al. (2013). Therefore, we expect that the CBP rescue protocol would also rescue the LTF deficit induced by CREB1 knockdown. This robustness of rescue protocols presumably occurs because CREB1 and CBP have similar regulatory roles in the CREB signaling pathway, in that CBP is an obligatory cofactor for CREB1. More generally, however, molecular deficits in different molecular pathways would be likely to require distinctively different rescue protocols chosen to interact with the dynamics of the affected pathway in such a manner as to restore, or optimize, pathway output.

Combining a pharmacological approach with a computationally designed rescue protocol

We also examined whether concurrent enhancement of PKA activity by rolipram could further increase the likelihood of successful rescue of impaired LTF by the rescue protocol alone. We found empirically that pairing rolipram with the rescue protocol significantly enhanced LTF relative to that produced by the rescue protocol alone. Rolipram alone did not produce LTF, perhaps because basal levels of cAMP synthesis did not produce sufficient PKA activation. Combination drug

therapies that may improve memory and cognition are being investigated for Alzheimer's disease (Aytan et al., 2013; Kawahara et al., 2014) and schizophrenia (Stenberg et al., 2011; Xiao et al., 2012). When developing combined drug therapies, a desirable goal is to obtain synergism, in which the efficacy of paired drugs exceeds the efficacy that would be predicted by summing the

effect of the individual drugs (Loewe, 1953; Novick, 2013; Zhang et al., 2014). Dose reductions facilitated by synergism may also minimize undesirable off-target effects of the drugs (Zimmermann et al., 2007). An extension of this idea is to combine the use of enhanced training protocols with one or more pharmacological agents. Consequently, we used simulations to predict whether an analogous form of synergism might exist between rolipram and the rescue stimulus protocol. Would the rescue of LTF (i.e., increase in the peak pC/EBP level) due to concurrent application of rolipram and the rescue protocol exceed the sum of the increases produced by these treatments individually? Only a small degree of synergism was predicted by these simulations. Nonetheless, it is likely that replacing the standard protocol with a rescue protocol would reduce the dosage of rolipram required for complete rescue of LTF, thereby reducing off-target drug effects. The relatively weak synergistic effects of rolipram and training may be due to the fact that the dynamics of the signaling cascade leading to phosphorylation of C/EBP, in which increases in cAMP levels enhance PKA activity and, in parallel, ERK is activated, do not appear to be governed by substantial feedback interactions, at least for the protocols used here. Greater synergistic interactions with pharmacological treatments are likely to occur when more complex biochemical elements, such as feedback loops, play a substantial dynamic role (Yin et al., 2014; Zhang et al., 2014). Feedback and cross-talk interactions affecting CREB1 and CREB2 expression do play a role at later times, during the consolidation phase of LTF (Mohamed et al., 2005; Song et al., 2007; Liu et al., 2011a), but they may not contribute to the synergism exhibited by the protocols used here to induce LTF.

Limitations of the model

Although simulations predicted that the rescue protocol would completely restore LTF impaired by CREB1 knockdown (Fig. 2*B*; taking peak pC/EBP as a predictor for the amount of LTF), subsequent experiments showed that the rescue protocol only partially rescued the impaired LTF (Fig. 4). This discrepancy is likely due to the fact that the current model is incomplete. It does not include the intermediate and long-term active phases of PKA, the persistent active phase of ERK, nor the activation of protein kinase C ζ or protein kinase M, all of which contribute to the cascade of events underlying LTF (Müller and Carew 1998; Sharma et al., 2003; Cai et al., 2011). For example, CREB1 itself is

Figure 6. Rolipram enhanced the effectiveness of the rescue protocol in restoring impaired LTF. *A*, Representative traces of EPSPs immediately before (pretest) and 24 h after (post-test) the rescue protocol. SNs were injected with CREB1 siRNA 72 h before the pretest. Restoration of LTF by rolipram and/or the rescue protocol was examined in three treatment conditions: (1) the rescue protocol with rolipram (black trace), (2) the rescue protocol without rolipram (red trace), and (3) vehicle treatment with rolipram (blue trace). *B*, Summary data. Error bars represent the mean $\pm$ SEM. A one-way ANOVA indicated no significant differences in the amplitude of pretest EPSPs among these groups ($F_{(2,27)} = 0.87, p = 0.43$). * $p < 0.05$.

upregulated during the consolidation phase, and this upregulation contributes to LTF (Liu et al., 2011a). Consequently the empirical knockdown of CREB1 with siRNA would not only reduce basal levels of CREB1, but also its late induction, with this

later effect not captured by the model. Thus, it is likely that empirically the rescue protocol did not completely compensate for impairment of CREB1 function at these later times. In addition, the dynamics of CREB2 are regulated (Liu et al., 2011b; Rajasethupathy et al. 2012). Variants of the canonical cAMP response element, to which CREB1 and CREB2 bind, are present in the *creb1* and *creb2* promoters (Mohamed et al., 2005), suggesting that both positive and negative feedback loops play a role in CREB1 and CREB2 dynamics. More precise prediction of rescue protocols may be feasible after the model is extended to include these feedback loops and other processes underlying the induction and consolidation of LTF.

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