Research

Superior long-term synaptic memory induced by combining dual pharmacological activation of PKA and ERK with an enhanced training protocol

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Developing treatment strategies to enhance memory is an important goal of neuroscience research. Activation of multiple biochemical signaling cascades, such as the protein kinase A (PKA) and extracellular signal-regulated kinase (ERK) pathways, is necessary to induce long-term synaptic facilitation (LTF), a correlate of long-term memory (LTM). Previously, a computational model was developed which correctly predicted a novel enhanced training protocol that augmented LTF by searching for the protocol with maximal overlap of PKA and ERK activation. The present study focused on pharmacological approaches to enhance LTF. Combining an ERK activator, NSC, and a PKA activator, rolipram, enhanced LTF to a greater extent than did either drug alone. An even greater increase in LTF occurred when rolipram and NSC were combined with the Enhanced protocol. These results indicate superior memory can be achieved by enhanced protocols that take advantage of the structure and dynamics of the biochemical cascades underlying memory formation, used in conjunction with combinatorial pharmacology.

One system amenable to a detailed analysis of memory at the cellular level is LTF of the monosynaptic connections between sensory neurons (SN) and motor neurons (MN) of *Aplysia* (Kandel 2001). LTF is induced by in vivo training, and correlates with long-term sensitization (LTS), a form of LTM (Frost et al. 1985; Cleary et al. 1998). LTF can be mimicked in vitro by application of 5-HT (Montarolo et al. 1986; Emptage and Carew 1993; Zhang et al. 1997). Two cascades required for LTF mediate activation of PKA (Schacher et al. 1988; Müller and Carew 1998; Chain et al. 1999) and the MAP kinase isoform termed ERK (Michael et al. 1998; Sharma et al. 2003; Sharma and Carew 2004). These kinases phosphorylate transcription factors (CREB1 for PKA, CREB2 for ERK) (Bartsch et al. 1995, 1998) leading to induction of genes essential for LTF (Lee et al. 2008; Alberini 2009). Previously, we used a computational model to identify a novel, irregularly spaced training protocol that maximized PKA/ERK interactions. Empirical studies verified this "Enhanced" protocol increased LTF and improved LTS (Zhang et al. 2012). Moreover, we found that computationally designed training protocols rescue deficits in LTF due to molecular lesions in the signaling cascades mediating LTF (Liu et al. 2013; Zhou et al. 2015).

The requirement for PKA and ERK suggests that combined pharmacological activation of these kinases could substantially enhance LTF and long-term memory (LTM). We used rolipram, an inhibitor of cAMP phosphodiesterase (PDE), to activate PKA. Rolipram has improved long-term potentiation (LTP) and LTM in normal rodents and in rodent models of Rubinstein–Taybi syndrome and traumatic brain injury (Bourtchouladze et al. 2003; Titus et al. 2013). To activate ERK, we focused on inhibitors of dual-specificity phosphatases (DUSPs) that inactivate MAPKs. DUSP6 (also termed MKP3) is 100-fold more active toward ERK2, which mediates LTF, than toward the MAPK isoforms c-Jun amino-terminal kinase (JNK) and p38 MAPK (Groom et al. 1996). NSC295642 (NSC) is a specific DUSP6 inhibitor (Vogt et al. 2003). The specificity of DUSP6 for ERK2 suggests that NSC might promote LTF, and not LTD which is facilitated by p38 MAPK (Guan et al. 2003). We proceeded to test the hypothesis that LTF could be further intensified by combining NSC and rolipram with an enhanced training protocol.

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Results

NSC selectively activates ERK and potentiates 5-HT-induced ERK phosphorylation

We located a predicted protein in the *Aplysia* genome (NCBI: XP_005099472.1) with a dual specificity phosphatase domain (NCBI: cd00127) and an amino-terminal regulatory domain typical of an ERK2 phosphatase (NCBI: cd01446) (Marchler-Bauer et al. 2015). Multiple sequence alignment using Clustal Omega (Soding 2005; Blackshields et al. 2010) showed greatest similarity to the subfamily of human DUSPs that preferentially targets ERK (Fig. 1A). This observation gave us a rationale to seek a concentration of NSC that would increase ERK phosphorylation without affecting p38 MAPK, because ERK and p38 MAPK play opposing roles in synaptic plasticity. ERK contributes to LTF by phosphorylating CREB2, releasing its repression of CREB1-mediated transcription (Bartsch et al. 1995; Lee et al. 2008). p38 MAPK activity contributes to long-term depression (LTD) by phosphorylating CREB2 at a different site, enabling CREB2 to inhibit CREB1-mediated transcription (Guan et al. 2002, 2003). p38 MAPK also inhibits ERK in *Aplysia* (Fioravante et al. 2006). We used immunofluorescence to measure activated (phosphorylated) ERK (pERK) levels and activated (phosphorylated) p38 MAPK (p-p38 MAPK) in isolated SNs after 2 h of NSC incubation.

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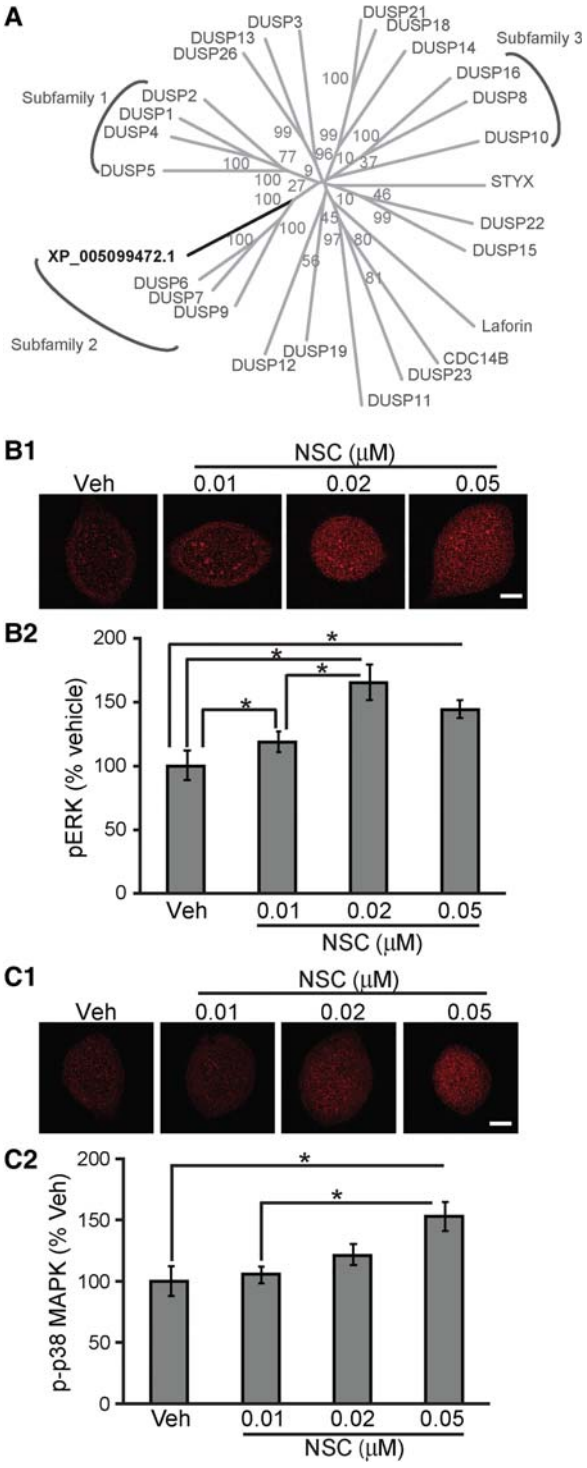

Figure 1. A low NSC concentration specifically activates ERK but not p38 MAPK. (*A*), Cladogram depicting the relative distance of the predicted protein sequence of *Aplysia* DUSP (NCBI: XP_005099472.1) from those of human DUSPs, constructed with QuickTree (Howe et al. 2002). The predicted protein shows greater similarity with proteins in subfamily 2. Bootstrap values are percentages based on 1000 replicates. (*B*1) and (*C*1) Representative confocal images of pERK and p-p38 MAPK immunofluorescence in SNs treated for 2 h with NSC at different concentrations. Scale bar, 20 μm. (*B*2 and *C*2) Summary data. In this and subsequent illustrations, error bars represent SEM and significant differences are indicated by * for $P < 0.05$.

Incubation with 0.01, 0.02, or 0.05 μM NSC led to an increase of 20 ± 8% ($n = 7$), 69 ± 12% ($n = 5$), and 45 ± 8% ($n = 5$), respectively, in levels of pERK compared with vehicle (Veh) controls ($n = 9$) (Fig. 1B2). A one-way repeated-measures (RM) ANOVA indicated significant overall differences among the groups ($F_{(3,25)} = 20.6$, $P < 0.001$). Post hoc comparisons (Tukey test) revealed a significant increase in pERK for all groups (0.01 μM: $P = 0.02$, 0.02 μM: $P < 0.001$, and 0.05 μM: $P < 0.001$) compared with Veh. 0.01 μM NSC increased pERK to a lesser extent than 0.02 and 0.05 μM NSC (0.02 versus 0.01 μM: $P = 0.006$; 0.05 versus 0.01 μM: $P = 0.006$).

Treatment with 0.02 or 0.05 μM NSC for 2 h led, respectively, to a nonsignificant 19 ± 8% change and to a 53 ± 12% increase in p-p38 MAPK compared with Veh, whereas 0.01 μM NSC had no evident effect (5 ± 7%, Fig. 1C2). A one-way RM ANOVA indicated significant overall differences among the groups ($n = 6$, $F_{(3,23)} = 8.88$, $P = 0.001$). Post hoc comparisons revealed the increase produced by 0.05 μM was significant ($P = 0.002$). These data indicate that at 0.01 and 0.02 μM, NSC selectively activates ERK but not p38 MAPK. Therefore, 0.01 μM was used to determine whether LTF could be augmented by NSC.

We also examined whether NSC could potentiate 5-HT-induced ERK activation. Isolated SNs were preincubated with NSC for 30 min, then given five pulses of 1 μM 5-HT (standard regularly spaced protocol, Materials and Methods) in the continued presence of NSC (2 h total). We used a low concentration (1 μM) of 5-HT, which produces a small (~20%) amount of LTF (Liu et al. 2013), to ensure the ERK cascade was well below saturation and thus sensitive to pharmacological effects. Immediately after treatment, SNs were fixed for immunofluorescence (Fig. 2A). Four groups of SNs were used: (1) Veh alone, (2) 5-HT alone, (3) NSC alone, and (4) 5-HT + NSC (Fig. 2B). Compared with Veh, 5-HT led to a nonsignificant 38 ± 13% change in pERK (Fig. 2C). pERK in the 5-HT + NSC group was increased by 91 ± 24% relative to Veh, and for the NSC alone group there was a nonsignificant change of 13 ± 6% relative to Veh. A one-way RM ANOVA indicated significant overall differences among the groups ($n = 6$, $F_{(3,23)} = 6.2$, $P = 0.006$). Post hoc comparisons indicated that although pERK was elevated by both 5-HT alone and NSC alone, these increases were not statistically significant, (5-HT versus Veh, $P = 0.262$; NSC versus Veh, $P = 0.607$). The lack of statistical significance of the NSC-induced increase in pERK is in contrast to the data in Figure 1B, in which pERK was significantly increased by 0.01 μM NSC. We believe the lack of statistical significance of the increase in Figure 2 is the consequence of the low concentration of NSC which may be near the minimum effective dose of ERK activation. In addition, the sample size used in that experiment was smaller ($n = 6$ for both control and NSC groups; whereas in Figure 1B, $n = 9$ for control and $n = 7$ for 0.01 μM NSC). However, 5-HT + NSC significantly increased pERK by 91 ± 24%, compared with Veh ($P = 0.006$) and to 5-HT or NSC alone (5-HT + NSC versus 5-HT, $P = 0.003$; 5-HT + NSC versus NSC, $P = 0.009$).

NSC alone and rolipram alone potentiate 5-HT-induced LTF

Aplysia LTF and mammalian LTP are dependent on ERK (English and Sweatt 1997; Michael et al. 1998; Kanterewicz et al. 2000; Sharma et al. 2003), thus selective activation of ERK by NSC might enhance synaptic memory. LTF was induced by a procedure used previously (Fig. 3A1). EPSPs were measured prior to five pulses of 5-HT treatment (pretest), and 24 and 48 h after treatment (posttest) in four groups: (1) Veh, (2) 5-HT (Standard protocol), (3) NSC alone, and (4) 5-HT + NSC (Fig. 3A2). 5-HT led to a 21 ± 6% ($n = 13$) increase in EPSP amplitude at 24 h and no change

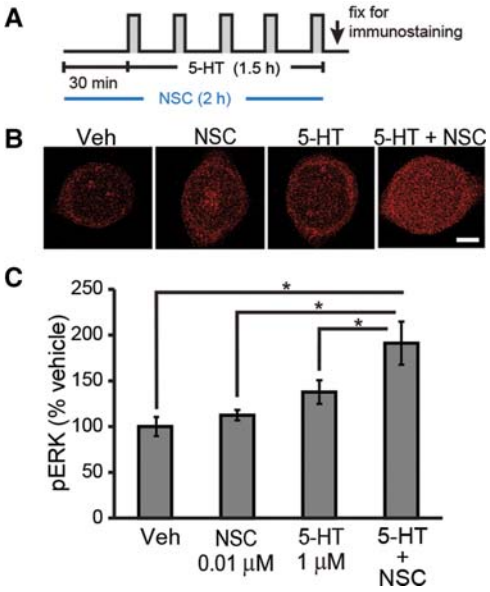

Figure 2. NSC and 5-HT activated ERK in cultured SNs. (*A*) Protocol for NSC application. (*B*) Representative confocal images of pERK immunofluorescence in SNs treated with 1 μM 5-HT or Veh in the presence or absence of 0.01 μM NSC. Scale bar, 20 μm. (*C*) Summary data. 5-HT +NSC gave a substantially greater increase in pERK than did NSC or 5-HT alone.

(−4 ± 5%) ($n = 9$) 48 h after treatment (Fig. 3A3). NSC had a pronounced effect on LTF. The EPSP increase in the 5-HT + NSC group was 42 ± 9% ($n = 9$) at the 24 h post-test and 40 ± 10% ($n = 6$) at 48 h. A two-way ANOVA indicated significant overall differences among the groups ($F_{(3,65)} = 8.82$, $P < 0.001$). However, post hoc comparisons of the main effects revealed neither 5-HT alone nor NSC alone significantly induced LTF (5-HT versus Veh, $P = 0.449$; NSC versus Veh, $P = 0.632$). The lack of significance of a main effect of LTF by 5-HT alone was presumably due to the decay, by 48 h, of the initial EPSP increase at 24 h. However, the combination of 5-HT with NSC yielded significant LTF (5-HT + NSC versus Veh, $P < 0.001$), greater than that produced by NSC alone or 5-HT alone (5-HT + NSC versus NSC, $P < 0.001$; 5-HT + NSC versus 5-HT, $P < 0.001$).

Previously we combined 0.2 μM rolipram with a training protocol computationally predicted to enhance LTF. The combination restored LTF impaired by siRNA-mediated knock down of CREB1 (Zhou et al. 2015). This result motivated us to examine whether 0.2 μM rolipram enhanced normal LTF. EPSPs were measured prior to 5-HT (Standard protocol) treatment, and 24 and 48 h after treatment (Fig. 3B1). Four groups were used: (1) Veh, (2) 5-HT, (3) rolipram (Roli) alone, and (4) 5-HT + Roli (Fig. 3B2). 5-HT alone led to only a 17 ± 3% ($n = 10$) increase in EPSP amplitude at 24 h and no change (6 ± 10%, $n = 9$) at 48 h (Fig. 3B3). However, the increase in the 5-HT + Roli group was 31 ± 6% ($n = 8$) at 24 h and 27 ± 11% ($n = 8$) at 48 h. Roli alone led to an 18 ± 6% ($n = 10$) increase at 24 h and to a 10 ± 4% ($n = 10$) increase at 48 h. The EPSP amplitude at 24 h in the Veh group was not changed (1 ± 3 %, $n = 9$) and was slightly increased at 48 h (13 ± 9%, $n = 8$). A two-way ANOVA indicated significant overall differences among the groups ($F_{(3,59)} = 7.78$, $P < 0.001$). Post hoc comparisons of the main effects revealed 5-HT alone or Roli alone induced significant synaptic enhancement (5-HT versus Veh, $P = 0.018$; Roli versus Veh, $P = 0.016$). Significant differences were observed between the 5-HT + Roli group and the rolipram alone or 5-HT alone groups (5-HT + Roli versus Roli, $P = 0.032$; 5-HT + Roli versus 5-HT, $P = 0.041$). Thus, rolipram potentiated 5-HT-induced LTF.

Combining NSC and rolipram yields greater enhancement of LTF

To determine whether NSC and rolipram can further enhance LTF, SN-MN cocultures were preincubated with NSC and/or rolipram for 30 min, then treated with the Standard protocol of five pulses of 5-HT in continued drug presence (Fig. 4A). EPSP amplitudes in response to 5-HT alone were increased by 21 ± 6% ($n = 10$) relative to Veh at 24 h and not changed (−9 ± 7%, $n = 9$) at 48 h after treatment (Fig. 4C). The increase in the 5-HT + NSC group was 37 ± 4% ($n = 10$) at 24 h and 11 ± 4% ($n = 9$) at 48 h, and the increase in the 5-HT + Roli group was 35 ± 6% ($n = 8$) at 24 h and 15 ± 8% ($n = 7$) at 48 h. The increase in the 5-HT + NSC + Roli

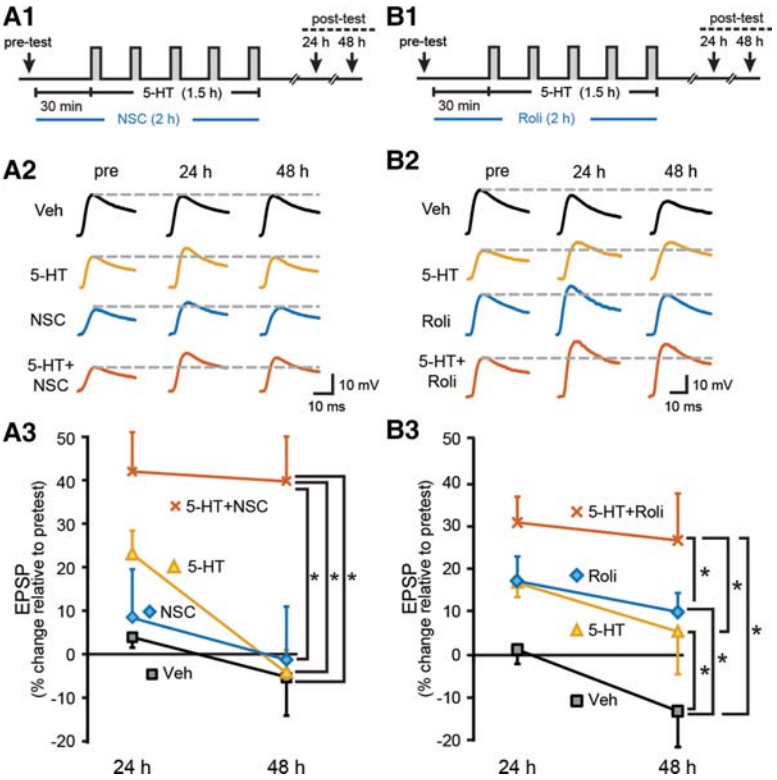

Figure 3. NSC or rolipram increases 5-HT-induced LTF. (*A1*) Protocol for NSC or rolipram application. (*A2*) NSC enhanced 5-HT-induced LTF. Representative EPSPs recorded before (pretest) and 24 or 48 h after (post-test) treatment with 5-HT, drugs, or Veh. Dashed lines represent the amplitude of the pretest EPSP. (*A3*) Summary data. NSC augmented 5-HT induced LTF. (*B1*–*B3*) Augmentation of LTF by rolipram. Panels *B1*–*B3* are analogous to *A1*–*A3*.

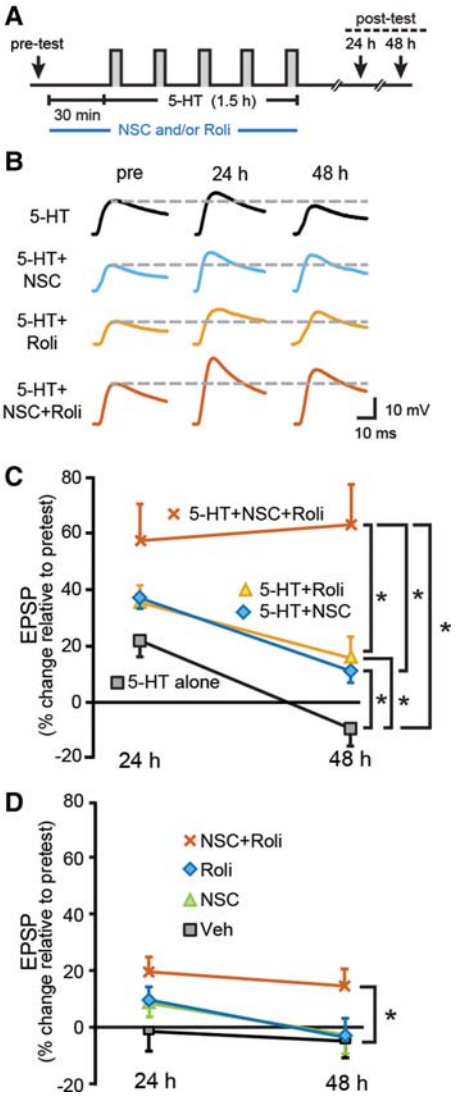

Figure 4. Coapplication of NSC and rolipram further increased LTF. (*A*) Protocol for combining NSC and rolipram applications. (*B*) Representative EPSPs. (*C*) Summary data. Coapplying NSC and rolipram potentiated LTF and also increased its persistence. (*D*) Combining NSC and Roli gave a small increase in EPSP amplitude.

group was 57 ± 13% ($n = 8$) at 24 h, and this relatively large increase persisted to 48 h (63 ± 14%, $n = 7$). A two-way ANOVA indicated significant overall differences among the groups ($F_{(3,61)} = 15.9$, $P < 0.001$). Post hoc comparisons of the main effects revealed a significant difference between 5-HT alone and either 5-HT + NSC or 5-HT + Roli (5-HT + NSC versus 5-HT, $P = 0.015$; 5-HT + Roli versus 5-HT, $P = 0.033$), and LTF induced by the drug combination and 5-HT was significantly greater than LTF induced by 5-HT with either individual drug (5-HT + NSC + Roli versus 5-HT + NSC, $P < 0.001$; 5-HT + NSC + Roli versus 5-HT + Roli, $P < 0.001$).

Control experiments examined the extent to which potentiation of LTF by NSC + Roli could result from EPSP increases due to the drugs without 5-HT. SN-MN cocultures were incubated with NSC and/or rolipram for 2 h. EPSP amplitudes with Veh alone did not change significantly at 24 h (−2 ± 8%, $n = 7$) and 48 h (−5 ± 7%, $n = 7$) (Fig. 4D). Neither NSC nor Roli alone induced significant synaptic enhancement. For NSC + Roli, EPSPs increased by 21 ± 5% ($n = 9$) at 24 h and 16 ± 6% at 48 h ($n = 6$). A two-way ANOVA indicated significant overall differences among the groups ($F_{(3,53)} = 3.75$, $P < 0.05$). Post hoc comparisons revealed the only significant difference was between NSC + Roli and Veh ($q = 4.60$, $P = 0.011$). Thus NSC and rolipram can interact to enhance synaptic strength. However, this effect is relatively small compared with that of combining NSC + Roli with 5-HT. To examine whether the combination of 5-HT + NSC + Roli produced stronger LTF than did NSC + Roli alone, we performed a statistical comparison between these two groups using a two-way ANOVA. A significant overall difference was obtained for the main effect for treatment ($F_{(1,26)} = 16.54$, $P < 0.001$). Post hoc comparisons revealed that there was a significant difference between NSC + Roli and 5-HT + NSC + Roli ($q = 5.75$, $P < 0.001$).

LTF is further intensified by combining NSC and rolipram with an enhanced training protocol

We next asked whether LTF can be further augmented by combining these drugs with the Enhanced protocol of five, 5 min, irregular but precisely spaced 5-HT applications (Fig. 5A2; Zhang et al. 2012). For these experiments we also assessed LTF at 96 h post-test, given that the previous experiments (Figs. 3, 4) suggested NSC and rolipram increased the persistence of LTF. The change in EPSP amplitude in response to the Enhanced protocol was 35 ± 9% ($n = 7$) relative to Veh at 24 h, 13 ± 13% ($n = 7$) at 48 h, and −27 ± 14%, ($n = 4$) at 96 h (Fig. 5C). The change in EPSP amplitude in the Standard protocol + NSC + Roli group was 35 ± 6% ($n = 7$) at 24 h, 39 ± 13% ($n = 7$) at 48 h, and 26.7 ± 15.9% ($n = 7$) at 96 h. Enhanced + NSC + Roli had the greatest increase in EPSP amplitude with 59 ± 12% ($n = 8$) at 24 h, 48 ± 8% ($n = 8$) at 48 h, and 55 ± 16% ($n = 8$) at 96 h. A two-way ANOVA indicated significant overall differences among the groups ($F_{(4,54)} = 6.82$, $P = 0.002$). Post hoc comparisons of the main effects indicated that LTF in the Enhanced + NSC + Roli group was greater than either the Enhanced alone group or the Standard + NSC + Roli groups (Enhanced + NSC + Roli versus Standard + NSC + Roli, $P = 0.04$; Enhanced + NSC + Roli versus Enhanced, $P = 0.002$). Thus, coapplying NSC, rolipram, and the Enhanced protocol produces LTF that is superior to LTF produced by the drug combination alone or the Enhanced protocol alone.

CREB1 phosphorylation is increased by combining dual pharmacological activation of PKA and ERK with an enhanced training protocol

We hypothesized that the increase in LTF induced by combining NSC and rolipram with 5-HT may be due, at least in part, to increased phosphorylation of CREB1. By elevating cAMP, rolipram activates PKA, which can then phosphorylate Ser^{85} of CREB1 (Bartsch et al. 1998). In mammals, ERK activates kinases that phosphorylate the analogous CREB Ser^{133} site, in particular ribosomal S6 kinase (RSK) (De Cesare et al. 1998) and mitogen- and stress-activated kinase (MSK) (Arthur et al. 2004). In *Aplysia*, 5-HT activates RSK via MAP kinase (Philips et al. 2013). Thus coapplication of NSC and rolipram may additively enhance CREB1 phosphorylation. To test this hypothesis we examined pCREB1 changes 2 h after the end of treatment, a time point when pCREB1 was increased in 5-HT treated ganglia and isolated SNs (Liu et al. 2011). Four groups were used: (1) Veh, (2) NSC alone, (3) Roli alone, and (4) NSC + Roli (Fig. 6A2). Compared with Veh, treatment with NSC or Roli alone resulted in nonsignificant −4% ± 8% and 11% ± 4% changes in pCREB1 level, respectively. NSC + Roli yielded an 18% ± 5% increase. A one-way RM ANOVA indicated significant overall differences among the groups ($n = 6$, $F_{(3,23)} = 4.7$, $P = 0.017$). Post hoc comparisons indicated that

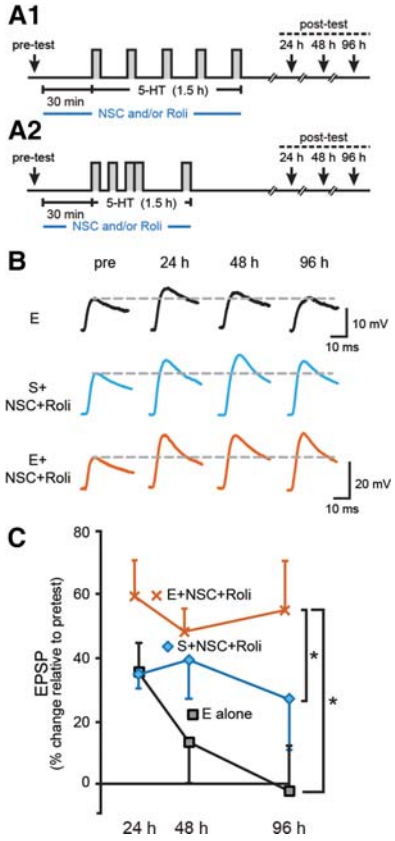

Figure 5. The Enhanced training protocol combined with NSC and rolipram was superior to the Standard protocol combined with these drugs. (*A1*) Protocol for Standard 5-HT treatment (S). (*A2*) Enhanced protocol (E). (*B*) Representative EPSPs. (*C*) Summary data. Application of NSC and rolipram together with the Enhanced 5-HT protocol produced LTF superior to that produced by the combination drug treatment alone or the Enhanced protocol alone.

pCREB1 was only increased significantly in the NSC + Roli group relative to Veh ($P = 0.03$). This increase may facilitate induction of LTF by 5-HT, and may also contribute to the small basal increase in EPSP amplitude produced by NSC + Roli (Fig. 4D).

Next, we examined whether the drug combination could enhance 5-HT-induced CREB1 phosphorylation. SNs were preincubated with NSC and/or rolipram for 30 min, then treated with the Standard protocol in continued drug presence. pCREB1 was measured 2 h after treatment in four groups: (1) Veh, (2) Standard, (3) NSC + Roli, and (4) Standard + NSC + Roli. The Standard protocol alone led to a 25 ± 7% increase in CREB1 phosphorylation compared with Veh (Fig. 6B2). NSC + Roli produced a 27 ± 8% increase, Standard + NSC + Roli produced a 44 ± 6% increase. A one-way RM ANOVA indicated significant differences among the groups ($n = 5$ experiments, $F_{(3,18)} = 13.9$, $P < 0.001$). Compared with Veh, the increases in pCREB1 observed in Standard alone and NSC + Roli alone were significant (Standard versus Veh, $P = 0.06$; NSC + Roli versus Veh, $P = 0.013$). A greater increase was observed in the Standard + (NSC + Roli) group compared with either the Standard alone or NSC + Roli alone groups (Standard + NSC + Roli versus S, $P = 0.029$; Standard + NSC + Roli versus NSC + Roli, $P = 0.032$). Thus, Standard + NSC + Roli elicits greater CREB phosphorylation than does Standard alone or the drug combination alone, which could help to explain the substantial LTF enhancement by Standard + NSC + Roli (Fig. 4C).

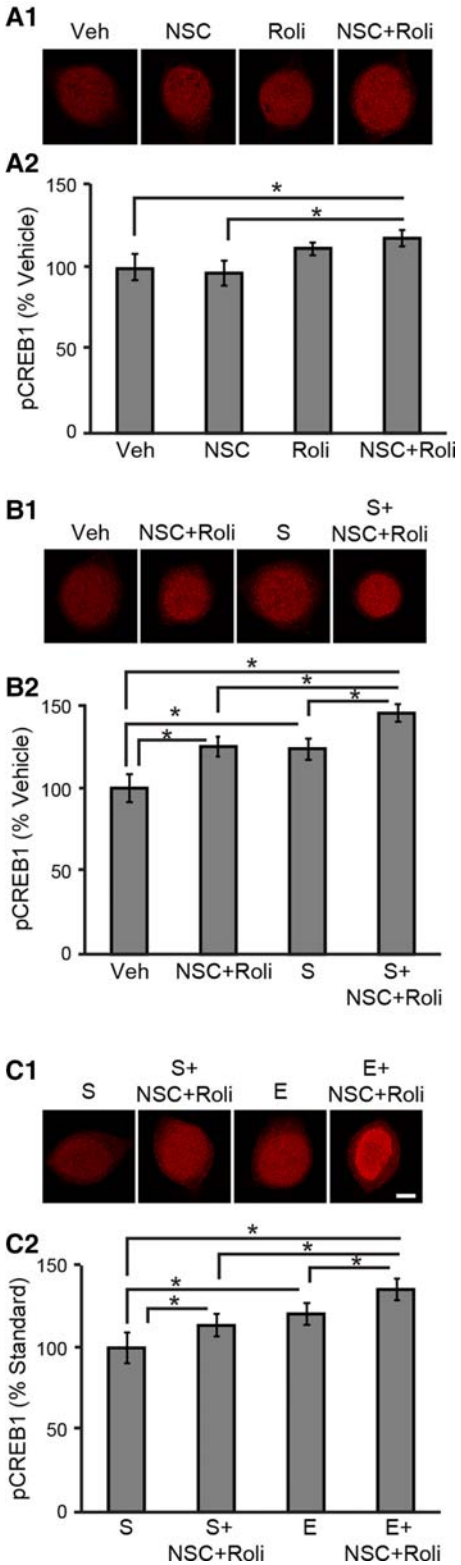

Figure 6. Activation of CREB1 in SNs. (*A1–A2*) Combining NSC and Roli led to significant increases in pCREB1. (*B1–B2*) 5-HT together with these drugs induced larger increases in pCREB1. (*C1–C2*) NSC and Roli, in combination with the Enhanced protocol (E) (Fig. 5A2), produced greater activation of pCREB1 than did E alone or the drugs in combination with the Standard protocol (S) (Fig. 5A1). Measurements were made 2 h after the end of treatment.

Finally, we examined CREB1 phosphorylation associated with the superior LTF produced by combining NSC and rolipram with the Enhanced training protocol. We predicted that increased LTF would correlate with increased pCREB1. Compared with the Standard protocol, the Enhanced protocol led to a 22 ± 6% increase in CREB1 phosphorylation. Also compared with Standard, the treatment with Standard + NSC + Roli led to a 15 ± 7% increase, whereas treatment with Enhanced + NSC + Roli led to a 36 ± 7% increase (Fig. 6C2). A one-way RM ANOVA indicated significant overall differences among the groups ($n = 6$ experiments, $F_{(3,23)} = 12.0$, $P < 0.001$). The increases in pCREB1 observed with Enhanced alone and with Standard + NSC + Roli were significantly larger than with Standard alone (Enhanced versus Standard, $P = 0.008$; Standard + NSC + Roli versus Standard, $P = 0.03$). A greater increase was also observed in the Enhanced + NSC + Roli group compared with either Enhanced alone or Standard + NSC + Roli (Enhanced + NSC + Roli versus Enhanced, $P = 0.032$; Enhanced + NSC + Roli versus Standard + NSC + Roli, $P = 0.009$). These results indicate that Enhanced + NSC + Roli activates CREB1 more than either the Enhanced protocol alone or the drugs in conjunction with the Standard protocol, suggesting the greater LTF induced by Enhanced + NSC + Roli (Fig. 5) is associated with increased CREB1 phosphorylation.

Discussion

Superior synaptic memory is achieved by an enhanced stimulus protocol together with combinatorial pharmacology

Treatments to improve human learning and memory have been explored using a wide variety of pharmacological agents (Lynch et al. 2014). Generally, the approach has been to focus on a single drug target such as histone deacetylase activity or AMPA receptor conductance (Alarcon et al. 2004; Baudry et al. 2012). However, targeting multiple pathways has clear advantages, allowing use of lower drug doses, which may reduce side effects (Smolen et al. 2014; 2016; Zhang et al. 2014). Although activation of PKA has been shown to ameliorate LTP and LTM deficits in rodent disease models (Balakrishnan et al. 2016; Bourtchouladze et al. 2003), few if any attempts have been made to improve LTM by activating the ERK cascade alone or in combination with other pathways. This is due at least in part to lack of an effective drug that activates the ERK cascade.

We used the DUSP6 inhibitor NSC 295642 to activate ERK without activating p38 MAPK. Importantly, we found that NSC works together with rolipram to increase LTF. Any other agents that specifically activate ERK might similarly be investigated as potential enhancers of plasticity and memory. Higher concentrations of NSC will activate p38 MAPK, which may in turn induce LTD. Thus, NSC's effective concentration range is limited. Nevertheless, the present study indicates the potential advantages of combining manipulations that target the PKA and ERK pathways. Moreover, a further increase in LTF was found when these drugs were combined with the Enhanced training protocol previously demonstrated to increase LTF and LTM.

In future work, it will be important to determine whether these pharmacological manipulations, possibly in conjunction with enhanced training protocols, can rescue cellular analogs of disease states with a deficit in LTF, for example, knockdown of CREB binding protein levels by siRNA in SN-MN cocultures (a model system for Rubinstein–Taybi syndrome, Liu et al. 2013).

Mechanisms of enhancement

Increased CREB1 phosphorylation appears to constitute part of the mechanism underlying the enhancement of LTF by rolipram, NSC and the Enhanced training protocol. Indeed, increased pCREB1 levels have been found to correlate with augmented LTF induced by the Enhanced 5-HT protocol (Zhang et al. 2012) and to enable induction of LTF by a single 5-min pulse of 5-HT (Bartsch et al. 1998). In addition, knockdown of pCREB1 levels by siRNA impairs LTF (Liu et al. 2011). We found that phosphorylation of CREB1 in response to NSC and rolipram (Fig. 6) was qualitatively similar to the response of LTF (Fig. 4). When coapplied with the Standard 5-HT protocol (S), the drug combination significantly increased pCREB1 and LTF. It is plausible, although not yet demonstrated, that with NSC present, greater activation of ERK leads to increased activity of RSK or MSK, and thus an augmented 5-HT response with greater CREB1 phosphorylation (Fig. 7). With the Enhanced protocol, the level of pCREB1 likewise correlates with the amplitude of LTF, with this protocol producing more pCREB1 and greater LTF than does the Standard protocol (Zhang et al. 2012), and also with Enhanced + NSC + Roli producing more pCREB1 (Fig. 6C) and greater LTF (Fig. 5) than Standard + NSC + Roli. The Enhanced protocol was previously predicted to maximize the overlap between PKA and ERK activities (Zhang et al. 2012), and given the plausible assumption that the amount of LTF correlates with the amount of concurrent, overlapping PKA and ERK activation, this protocol was further predicted to increase LTF compared with the Standard protocol. Rolipram and NSC act to increase PKA and ERK activities independently of 5-HT. Therefore, combining the Enhanced protocol with rolipram + NSC is expected to further increase overlap of PKA and ERK activities, with the dynamic activation of these kinases due to the Enhanced protocol being augmented by the static activation due to the drugs. Thus combining both drugs with the Enhanced protocol was predicted and observed (Fig. 5C) to lead to a further boost in LTF relative to the drugs alone, or to the Enhanced protocol alone.

The concentration (1 μM) of 5-HT used in the present study was selected to avoid ceiling and floor effects (Ghirardi et al. 1995; Liu et al. 2014). In addition, abundant evidence indicates that 1 μM 5-HT activates transcription factors and gene expression (Montarolo et al. 1986; Ghirardi et al. 1995). Consequently, the enhancement of LTF that we observed with agents that act to increase CREB1 phosphorylation is likely due to a graded rather than stepwise enhancement.

In support of a graded enhancement, NSC + Rolipram without 5-HT led to a smaller, yet significant phosphorylation of

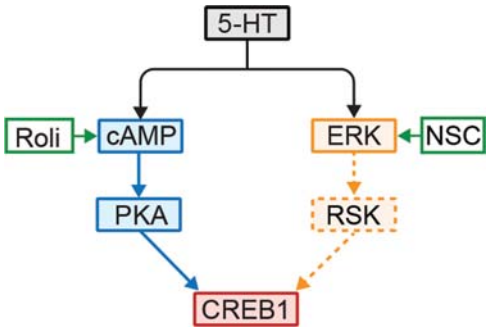

Figure 7. Model of the effects of NSC and rolipram on LTF. During LTF, 5-HT leads to elevation of cAMP levels, activating PKA, which phosphorylates and activates CREB1. 5-HT also activates the ERK cascade, and ERK may, via activation of RSK or MSK kinases, further activate CREB1 (dashed pathway). Activation of CREB1 induces transcription of *c/ebp* and other genes essential for LTF. Rolipram and NSC boost CREB1 activation by, respectively, amplifying PKA and ERK activities. The Enhanced training protocol boosts CREB1 activation by maximizing the overlap of PKA and ERK activities.

CREB1 (Fig. 6A) and also produced a small long-lasting increase in basal synaptic strength (Fig. 4D) as did rolipram alone (Fig. 3B). These small increases in basal synaptic strength may not be behaviorally relevant. However, when boosted further by training, the facilitated EPSP may have a greater effect in driving the postsynaptic neuron due to the nonlinear input–output relationship of EPSPs and action potential firing. One difference between the measurements of pCREB1 and synaptic facilitation was that the Standard protocol + NSC + Roli produced a greater increase in LTF, but not a greater increase in pCREB1, compared with the Enhanced protocol (Figs. 5C, 6C). This result suggests that NSC + Roli also increases activation of additional kinases not assessed here.

Indeed, other kinase targets are known to be essential for LTF and are likely to contribute to augmentation of LTF by the Enhanced protocol and by NSC + Roli. For example, as illustrated in a model schematic (Fig. 7), PKA phosphorylates and activates CREB1, inducing expression of genes that are essential for LTF and are regulated by cAMP response elements, which bind CREB1. *creb1* itself is one such gene. Induction of *creb1* transcription by CREB1 leads to increased CREB1 levels, and thereby further induction of *creb1* and other genes. These interactions form a positive feedback loop that enhances and prolongs CREB1 expression, and that is necessary for consolidation of LTF (Liu et al. 2011).

In this connection, it is interesting to note that the effects of the Enhanced protocol and of NSC and rolipram appear relatively larger (greater percent increase relative to 5-HT without drugs) at later times (e.g., 48 h post-test) (Figs. 3–5; also evident previously, Liu et al. 2013). Stronger activation of the CREB1 feedback loop by the Enhanced protocol, or by 5-HT together with drugs, may lead to a longer effective duration of positive feedback, contributing to these relatively greater effects at later times. Because *c/ebp* is induced by CREB1 and repressed by CREB2 (Guan et al. 2002) and C/EBP is phosphorylated by ERK (Yamamoto et al. 1999), the PKA and ERK signaling pathways also converge to enhance the level and phosphorylation of C/EBP, a transcription factor critical for LTF (Alberini et al. 1994).

Could our simplified computational model, with a limited number of molecular components, predict interventions that rescue LTF impaired by deficits in activity of molecules not included? For example, several additional kinases contribute to LTF, such as phosphatidylinositol 3-kinase (Hu et al. 2006) and PKC and PKMζ (Cai et al. 2011; Hu et al. 2011). We believe that a boost of the amounts of concurrent PKA activation and ERK activation (e.g., by the combined drug treatment, and/or the Enhanced 5-HT protocol) could compensate, at least in part, for reductions in the activity of these additional kinases, because PKA and ERK activation are likely not saturated by 1 μM 5-HT.

Implications

Activation of PKA, ERK, CREB, and C/EBP is necessary for at least some forms of LTP (e.g., hippocampal LTP of CA3-CA1 connections), and LTM, in diverse species (English and Sweatt 1997; Kanterewicz et al. 2000; Patterson et al. 2001; Pittenger et al. 2002; Abel and Nguyen 2008; Peters et al. 2009; Bambah-Mukku et al. 2014; Hell 2016). Thus, drug combinations activating these pathways may constitute candidates for enhancing human synaptic plasticity, learning, and memory. Ampakines are another class of drugs that may be effective for augmentation of learning (Lauterborn et al. 2016). It would be of interest to examine the efficacy of treatments that combine an ampakine with an activator of ERK, PKA, or both. In addition, improved spaced training protocols that enhance learning in humans and other animals are being explored (Philips et al. 2013; Seese et al. 2014; Vlach et al. 2014; Smolen et al. 2016). The results presented here suggest further investigation of the effects of combined drugs, alone or in conjunction with training protocols predicted to be efficacious, may lead to effective treatments for cognitive impairments.

Materials and Methods

Animals and cell cultures

All experiments used primary cell cultures of identified cells from *Aplysia*, which were obtained from the University of Miami NIH National Resource for *Aplysia*. Isolated sensory neurons (SNs) or sensory neuron–motor neuron (SN–MN) cocultures were prepared according to conventional procedures (Zhang et al. 2012). SNs were isolated from the ventral–caudal cluster of the pleural ganglion. The SNs in all the groups in each experiment repetition were obtained from the same sensory neuron cluster from a single animal. MNs used in coculture were isolated from the abdominal ganglion of juvenile animals. Of note, 5–10 SNs were plated on each dish. Dishes of SN–MN cocultures were plated with a single SN and a single MN. Both SN cultures and SN–MN cocultures were allowed to grow for 5 d at 18°C, and the growth medium was replaced prior to treatments and recordings with a solution of 50% L15 and 50% artificial seawater (ASW, containing 450 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 30 mM $MgCl_2$, 10 mM HEPES at pH 7.6).

Pharmacological treatment

The use of NSC in *Aplysia* has not been reported. Concentrations of NSC used in mammalian cell lines range from 0.01 to 2 μM (Vogt et al. 2003). In a pilot study we found that concentrations as low as 0.1 μM activated both ERK and p38 MAPK (data not shown). Therefore, we examined lower concentrations of NSC (i.e., 0.01, 0.02, and 0.05 μM) to search for a concentration which specifically activates ERK but not p38 MAPK.

Repeated applications of 5-HT induce LTF and ERK activation (Sharma et al. 2003; Sharma and Carew 2004). The Standard 5-HT protocol is to treat SN cultures or SN-MN cocultures with five, 5-min pulses of 1 μM 5-HT (Sigma-Aldrich) with a uniform interstimulus interval (ISI) of 20 min (onset to onset). The Enhanced training protocol, in contrast, is comprised of five 5-min pulses of 1 μM 5-HT with ISIs of 10, 10, 5, and 30 min. Control SN–MN cocultures were treated with an equal number of pulses of vehicle (Veh) (L15:ASW). For drug treatment, cocultures were exposed to NSC, rolipram, or a combination of NSC and rolipram, for 30 min before and throughout the Standard or Enhanced protocols.

Immunofluorescence

SNs were fixed for immunofluorescence immediately after treatment. A standard procedure was followed (Chin et al. 1999). Briefly, cells were fixed in a solution of 4% paraformaldehyde in PBS containing 20% sucrose for 30 min. After three rinses in PBS, fixed cells were blocked for 30 min at room temperature in Superblock buffer (Pierce) and 0.2% Triton X-100/3% normal goat serum, then incubated overnight at 4°C with anti-phosphorylated ERK (anti-pERK) antibody (1:500), anti-phosphorylated p38 MAPK (anti-p-p38 MAPK) antibody (1:200), or anti-phosphorylated CREB1 (anti-pCREB1, 1:500). Anti-pERK and anti-p-p38 MAPK antibodies were purchased from Cell Signaling. The anti-pCREB1 antibody was raised by a commercial vendor (Genemed Synthesis, Inc.) against the Ser^{85}-phosphorylated versions of the bovine serum albumin-conjugated CREB1 peptide KKRREILTRRPSYRK. Secondary antibody (goat anti-rabbit secondary antibody conjugated to Cy-3, Jackson Lab, 1:200 dilution) was applied to the cells for 1 h at room temperature. Cells were then mounted for confocal imaging. The intensity of staining was quantified in images obtained with a Zeiss LSM510 confocal microscope using a 63× oil immersion lens as described previously (Liu et al. 2011, 2013). A *z*-series of optical sections through the cell body (0.5 μm increments) was taken, and the

section through the middle of the nucleus was used for analysis of mean fluorescence intensity with Metamorph Offline software (Universal Imaging Corporation). All the neurons on each coverslip were analyzed, and measurements from these neurons were averaged. Therefore, the number of samples for the immunofluorescence studies represents the number of coverslips. In each experiment in Figures 1, 2, and 6, four groups of SNs from the same animal were used for different treatments. Groups corresponding to different treatments were processed concurrently, not sequentially, and these experiments were performed in a blind manner so that the investigator applying the treatments and analyzing the images was unaware of the treatment the SNs received.

Electrophysiology

Stimulation of presynaptic SNs was performed extracellularly using a blunt patch electrode filled with L15:ASW. Intracellular recordings from MNs were made with 10–20 MΩ sharp electrodes filled with 3 M potassium acetate connected to an Axoclamp 2-B amplifier (Molecular Devices). Data acquisition and analyses of resting potential, input resistance, and EPSP amplitude were performed with pCLAMP 10 software (Molecular Devices). Before measurement of EPSPs, MNs were held at −90 mV. Several measurements were made, including resting potential, input resistance of the MN, and EPSP amplitude. Cultures were excluded from further use if pretreatment measurements of EPSP amplitudes were <10 mV, larger than 30 mV, or sufficiently large to trigger an action potential. MNs with resting potentials more positive than −40 mV or input resistances <10 MΩ were also excluded. These measurements were repeated 24 and 48 h later to monitor long-term changes in synaptic strength. In some experiments, recordings were also repeated 96 h after treatment to examine the persistence of LTF. In cases where the post-test EPSP amplitude was accompanied by an action potential, the EPSP amplitude was assigned a value of 45 mV, which was the largest synaptic potential obtained in our culture system that did not trigger an action potential (Liu et al. 2011, 2013). Statistical analyses (one-way ANOVA) indicated that the amplitudes of EPSPs before treatment (pretest) for each group were not significantly different (Fig. 3A3: $F_{(3,46)} = 0.16$, $P = 0.92$; Fig. 3B3: $F_{(3,33)} = 0.82$, $P = 0.49$; Fig. 4C, $F_{(3,32)} = 0.51$, $P = 0.68$; Fig. 4D, $F_{(3,33)} = 0.06$, $P = 0.98$; Fig. 5C, $F_{(3,24)} = 0.89$, $P = 0.46$). Therefore, the amplitudes of the EPSPs measured after treatment (post-test) were normalized to those measured at pretest. The number of samples (*n*) reported in Results indicate number of cocultures. In each, SN–MN cocultures were randomly assigned to different treatments in an experiment. Groups corresponding to the different treatments were examined concurrently. The electrophysiological experiments were performed in a blind manner so that the investigator performing the experiments and analyzing the synaptic responses was unaware of the treatment the neurons received.

Statistical analyses

SigmaPlot version 11 (Systat Software, Inc.) was used to perform all statistical analyses. For immunocytochemistry, SNs were isolated from the same animal in each experiment repetition. Therefore, repeated measures statistics were used on unnormalized data, as done previously (Liu et al. 2008, 2011, 2013). For electrophysiological experiments, the amplitudes of the EPSPs measured 24 and 48 h after 5-HT treatment (post-test) were normalized to those measured at pretest. Data are presented as means ± SEM, and $P < 0.05$ was considered to represent statistical significance.

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