Computational design of enhanced learning protocols

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Learning and memory are influenced by the temporal pattern of training stimuli. However, the mechanisms that determine the effectiveness of a particular training protocol are not well understood. We tested the hypothesis that the efficacy of a protocol is determined in part by interactions among biochemical cascades that underlie learning and memory. Previous findings suggest that the protein kinase A (PKA) and extracellular signal–regulated kinase (ERK) cascades are necessary to induce long-term synaptic facilitation (LTF) in Aplysia, a neuronal correlate of memory. We developed a computational model of the PKA and ERK cascades and used it to identify a training protocol that maximized PKA and ERK interactions. In vitro studies confirmed that the protocol enhanced LTF. Moreover, the protocol enhanced the levels of phosphorylation of the transcription factor CREB1. Behavioral training confirmed that long-term memory also was enhanced by the protocol. These results illustrate the feasibility of using computational models to design training protocols that improve memory.

In the field of experimental psychology, virtually all of the learning protocols used in animal and human studies were developed on an ad hoc, trial-and-error basis. For example, multiple training trials spaced over time are generally more effective than multiple trials massed together at producing long-term memory (LTM)1. However, it is not known why one procedure is better than another, nor is the optimal spacing of trials known a priori. In principle, one way to enhance learning and memory is to design training protocols that are synchronized or in phase with the dynamics of biochemical cascades underlying the induction of LTM2,3. However, this task is challenging because of nonlinear interactions and multiple timescales in these cascades. One way of overcoming this challenge is to develop computational models of these nonlinear interactions and use the models to predict training protocols that maximize LTM.

Long-term sensitization (LTS) of withdrawal reflexes in the mollusk Aplysia is an example of LTM that is particularly well characterized4 and therefore serves as an excellent system for testing the concept that computational approaches can help design training protocols that enhance learning and memory. Distinct phases of memory for sensitization are induced by different numbers of training trials5–9. At the cellular level, LTS is a result, in part, of serotonin (5-HT)-induced LTF of synaptic connections between sensory neurons and motor neurons5,10,11. Two biochemical cascades required for LTF are those that mediate activation of PKA12–15 and the MAP kinase isoform ERK16–18. These kinases are both needed to phosphorylate transcription factors that are critical for LTF (for example, the transcriptional activator cAMP responsive element binding protein 1, CREB1)19,20. These events, in turn, induce genes whose products are essential for LTF21,22. The requirement for both PKA and ERK suggests that the strength of LTF is likely to depend on synergism between these kinases. Following a single 5-HT stimulus, PKA is activated rapidly, but transiently, with activity returning to near basal levels within 15 min13. In contrast, ERK is activated more slowly, with maximal activation occurring about 45 min after a single trial2. These results indicate that a single stimulus will produce little overlap between PKA and ERK. However, they also suggest that multiple properly timed stimuli could increase the overlap and thus the synergism of the two cascades. We tested the hypothesis that a stimulation protocol maximizing the overlap would enhance learning and memory. We developed a computational model of the PKA and ERK cascades and used simulations of the model to predict a protocol that could enhance learning and memory. Subsequent in vitro and in vivo empirical studies confirmed that LTF and LTS were enhanced by the protocol.

RESULTS

Computational model of PKA and ERK cascades

We developed a simplified mathematical model of the dynamic activation of PKA and ERK (Fig. 1a). In the model, interactions between PKA and ERK cascades were represented by a variable termed inducer. Because activation of ERK and PKA are both required for LTF15,16, inducer was proportional to the product of PKA and ERK activities. The peak levels of inducer, which corresponded to the peak synergistic interaction between PKA and ERK, were hypothesized to predict the efficacy of stimulus protocols.

As a point of reference, the simulated peak level of inducer produced by five 5-min pulses of 5-HT with uniform 20-min interstimulus intervals (ISIs) was selected (Fig. 1b). This ‘standard’ protocol has been widely used in experimental studies since its introduction in 1986 (ref. 23). To identify a protocol that produced the highest peak level of inducer, we simulated 9,999 alternative protocols. Each protocol included five 5-min 5-HT stimuli, but the ISIs were chosen as multiples of 5 min, ranging from 5 to 50 min (Supplementary Movie 1). Thus, each of the four ISIs had ten possible values (that is, 104 total possible permutations, one of which represented the standard protocol). Simulation of these protocols revealed considerable variability in the peak level of inducer (Supplementary Fig. 1).

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The protocol that maximized the peak level of inducer (the enhanced protocol) consisted of non-uniformly spaced 5-HT applications with ISIs of 10, 10, 5, and 30 min. The peak level was ~50% higher than in the standard protocol (Fig. 1c). To the extent that the model captured the salient features of the dynamics of PKA and ERK and the extent to which the synergism between PKA and ERK activity is essential for LTF, the enhanced protocol was predicted to induce more LTF than the standard protocol.

Enhancement of LTF

Electrophysiological recordings from sensorimotor co-cultures were used to test our predictions. Three groups of preparations were examined: the standard and enhanced protocols (see Fig. 1b,c) and a vehicle (control) group that received five 5-min applications of solution without 5-HT (20-min ISIs). In each group, excitatory postsynaptic potentials (EPSPs) were measured before 5-HT treatment (pretest) and 1, 2, and 5 d after 5-HT treatment (post-tests). LTF was defined as a significant increase in the EPSP amplitude in either the standard or enhanced groups as compared with EPSPs measured in the control group (Fig. 2a,b).

Statistical analyses (two-way ANOVA) indicated that there were significant overall differences in the amplitude of EPSPs among the three treatment groups ($F_{2,73} = 22.06, P < 0.001$) and among the three time points ($F_{2,73} = 7.28, P < 0.002$). Post hoc pair-wise comparisons (Student–Newman–Keuls (SNK) tests) indicated that both the standard protocol ($q_2 = 4.49, n = 10, P < 0.003$) and enhanced protocol ($q_3 = 7.57, n = 11, P < 0.001$) induced LTF 1 d after 5-HT treatment as compared with vehicle controls ($n = 8$). The LTF that was induced by the standard protocol, however, did not persist beyond the first day (day 2, $q_3 = 2.54, n = 10$; day 5, $q_2 = 0.21, n = 9$), as compared with controls on day 2 ($n = 8$) and on day 5 ($n = 8$). In contrast, the LTF that was induced by the enhanced protocol persisted for up to 5 d (day 2, $q_3 = 5.21, n = 9, P < 0.002$; day 5, $q_2 = 3.41, n = 9, P < 0.02$), as compared with vehicle controls. In addition, post hoc analyses indicated that the enhanced protocol induced significantly greater LTF at all time points as compared with the standard protocol (day 1, $q_2 = 3.18, P < 0.03$; day 2, $q_2 = 2.89, P < 0.05$; day 5, $q_3 = 3.73, P < 0.03$).

Increased levels of CREB1 phosphorylation

The induction and consolidation of LTF in vitro depends on the activity of several transcription factors. For example, increased levels of CREB1 phosphorylation are associated with the induction and consolidation of LTF at sensorimotor synapses. Because the enhanced protocol induced greater and longer-lasting LTF, we hypothesized that it is associated with a greater level of CREB1 phosphorylation. This hypothesis was tested in sensory neuron cultures (Fig. 3).

Three groups of sensory neuron cultures were examined. One group was treated with the standard protocol, the second group with the enhanced protocol (as described in Fig. 2) and the third group with the control protocol. In each group, the level of CREB1 phosphorylation was measured immediately (0 h) or 18 h after treatment, and the values in the standard and enhanced groups were normalized to vehicle controls. Statistical analyses (two-way ANOVA) indicated overall significant differences between the two 5-HT protocols ($F_{1,18} = 30.12, P < 0.001$). Pair-wise comparisons (SNK tests) indicated that, compared with the standard protocol, the enhanced protocol induced significantly higher levels of CREB1 phosphorylation immediately ($q_2 = 6.94, n = 4, P < 0.001$), and 18 h after treatment ($q_2 = 3.69, n = 7, P < 0.02$).
of biochemical cascades could be essential for understanding why specific training protocols are more effective than others, and thereby help to guide the development of better learning protocols.

One of our goals was to develop training protocols that enhanced learning and memory. However, our model (Fig. 1a) is also compatible with previous empirical results in which a massed training protocol (25 min of continuous 5-HT) produced significantly less LTF than a spaced protocol (five 5-min pulses of 5-HT)29. Simulations of this massed protocol produced a peak level of inducer of only 0.00016 µM, which was at the low end of the distribution (Supplementary Fig. 1).

Thus, the model was able to predict submaximal performance, which helped to increase confidence in its predictive power.

Many training protocols were predicted to outperform the standard protocol (Supplementary Fig. 1). These results indicate that the improvement found with the enhanced protocol did not represent a narrow range of model parameter values, but occurred for various combinations of values. Such behavior is expected for robust models of physiological processes in which optima should not be sharply peaked at very specific parameter values.

In humans, a number of cognitive models have been proposed to explain the superiority of spaced training or practice1,30, but no consensus has emerged as to their usefulness or generality31. These models include variations in processing, encoding and consolidation. It is likely that these models have had limited predictive ability, at least in part, because they are not based on known neurobiological processes. In contrast, our model is based on well-characterized biochemical cascades in individual neurons that participate in the induction and expression of LTM.

Although our model is limited to a description of the dynamics of biochemical cascades associated with the initial induction of LTM and to relatively brief training sessions (for example, 1.5 h), it successfully predicted a protocol that enhanced synaptic strength and memory for at least 5 d after stimulation. These results confirm the notion that important aspects of the induction of LTM occur during the initial training phase. The model is undoubtedly incomplete, however. For example, the slow dynamics of additional biochemical cascades contribute to LTM (for example, changes in the synthesis of transcription factors such as CREB1 and CREB2)32,33. However, these slower processes were not considered in our model. In addition, the model did not include descriptions of postsynaptic protein kinase C33, upregulation of local protein synthesis in the vicinity of the synapse34 or synthesis of the peptide sensorin in response to retrograde signals35, which are involved in LTF. Finally, the model did not account for the differences in the time course and magnitude of LTF (Fig. 2) from those of LTS.
(Fig. 4). Several factors may contribute to this discrepancy, including a nonlinear transformation of LTF into motor neuron activity and plasticity at other loci in the neural circuit that contribute to the behavioral enhancement. An expanded model that includes the dynamics of additional biochemical cascades and neural circuit elements would presumably have greater predictive capability.

Our results indicate the feasibility of using computational methods to assist in the design of training procedures that enhance learning. It will be important to determine the extent to which the computational approaches can be applied to other memory systems and, ultimately, applied to training procedures used to improve human cognition.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

Y.Z. implemented the computational model and ran all simulations. R.-Y.L. performed the electrophysiological and behavioral experiments. L.J.H.C. helped design and supervise the behavioral experiments. R.-Y.L. and G.A.H. performed the immunofluorescence experiments. D.A.B. performed the statistical analyses and prepared the illustrations. P.S. and D.A.B. helped design and supervise the computational studies. J.H.B.H. supervised and contributed to all aspects of these studies. All of the authors discussed the results and contributed to the writing and editing of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Model development. The mathematical model for the activation of PKA and ERK (Fig. 1a) was modified from an earlier model\(^\text{39}\) of the signaling cascades underlying LTF by only considering the initial (protein-synthesis independent) steps in the induction of LTF. In addition, a time delay was added to the phosphorylation of Raf (see below) and the variable *inducer* was added.

PKA exists largely as an inactive holoenzyme (PKA\(_h\)) that consists of regulatory (PKA\(_r\)) and catalytic (PKA\(_c\)) subunits (Fig. 1a). In response to 5-HT treatment, [cAMP] increases, and cAMP binds to the regulatory subunit, leading to the release of active catalytic subunit. The basal level of 5-HT was 0 µM, and during a pulse of 5-HT, its concentration was transiently increased to 50 µM. The dynamics of cAMP following 5-HT treatment and the cAMP-dependent activation of PKA are described by equations (1–4).

\[
\frac{d[cAMP]}{dt} = \lambda [5-HT] - k_{b,cAMP}[cAMP]
\]  
(1)

\[
\frac{d[PKAR]}{dt} = k_{b,PKA}[PKAc][PKAR] - k_{f,PKA}[PKAR][cAMP]^2
\]  
(2)

\[
\frac{d[PKA]}{dt} = k_{f,PKA}[PKAR][cAMP]^2 - k_{b,PKA}[PKAc][PKAR]
\]  
(3)

\[
\frac{d[PKAc]}{dt} = k_{f,PKA}[PKAR][cAMP]^2 - k_{b,PKA}[PKAc][PKAc]
\]  
(4)

A particle swarm optimization algorithm\(^\text{39}\) was used to identify the values for model parameters that produced the best fit between model simulations and the time course of short-term PKA activation after 5-HT treatment\(^\text{13}\). The model was initially tested with a single 5-min pulse of 5-HT to confirm that such a stimulus elicited a transient activation of PKA and a delayed transient activation of ERK with little overlap and therefore little increase in *inducer* (data not shown). Similarly, little overlap was produced when two 5-min pulses of 5-HT were applied with an ISI (onset to onset) of 15 or 60 min (data not shown). A substantial overlap of PKA and ERK activity occurred with an ISI of 45 min (data not shown). Substantial overlap (Fig. 1b) was also produced by the standard five-pulse 5-HT protocol that is commonly used to empirically induce LTF (that is, five–pulse 5-min 5-HT treatment with uniform 20-min intervals between the start of the pulses\(^\text{12,41,43}\)). To determine whether other protocols could more effectively activate *inducer*, we simulated 9,999 alternative protocols. Each protocol included five 5-min 5-HT stimuli, but the ISIs were chosen as multiples of 5 min in the range of 5–50 min. Thus, each of the four ISIs had ten possible values (that is, 10\(^4\) total possible permutations, one of which represented the standard protocol; Supplementary Movie 1).

The variable *inducer* was used to quantify the amount of overlap between PKA and ERK activities. *Inducer* was an abstraction representing the synergistic interaction between PKA- and ERK-dependent regulation of gene expression that is necessary for the induction of LTF. Thus, *inducer* was proportional to the product of PKA and ERK activities (equation (13))

\[
*inducer = k_{inducer}[PKAc][ERK]^2
\]  
(13)

where \(k_{inducer} = 1 \mu M^{-1}\).

Although optimization algorithms are available, several factors indicated that optimization algorithms were not necessary in the present study. First, we only varied four parameters. These four parameters were the ISIs between the five pulses of 5-HT (see Supplementary Movie 1). Given this small number of free parameters, it is likely that our systematic search of the parameter space was sufficient. Second, our search of the ISI parameter space did not reveal sudden, large changes in simulated protocol efficiencies. Rather, peak levels of *inducer* varied smoothly as ISIs were varied over the range sampled in the present study (data not shown). Thus, it is unlikely that further refinement of the search would yield substantially better training protocols. Third, empirical considerations limit the temporal precision with which drugs can be applied and washed out. Thus, ISIs were limited to 5-min increments. For the present study, there was no additional benefit in examining finer increments that could not be experimentally tested. Finally, we were more concerned with demonstrating the feasibility of predicting training protocols that enhance memory than finding an exact optimum.

Simulations indicated that a protocol that enhanced LTS and LTF would need to satisfy two criteria: significant activation of PKA and ERK, and maximum overlap of these activities. The enhanced protocol was superior to the standard protocol because it induced a greater level of ERK and because the peak level of PKA and ERK activities induced by the fifth stimulus significantly overlapped with the peak level of ERK activation (Fig. 1b, c), thus maximizing the level of *inducer*.

Fourth-order Runge-Kutta integration was used for integration of differential equations with a time step of 3 s. No significant improvement in accuracy was determined after at least two simulated days, before any stimuli. The model was programmed in XPP\(^\text{42}\), and simulated on a Pentium 4 Duo Core processor microcomputer. Source codes are available upon request.

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Cell culture, 5-HT treatment and electrophysiology. For cellular experiments, *Aplysia californica* were obtained from the US National Institutes of Health *Aplysia* resource facility (University of Miami). Motor neurons were isolated from abdominal ganglia from juvenile animals (0.8–1.5 g), and sensory neurons were isolated from pleural ganglia from 60–100-g animals. Isolated sensory neurons or sensory neuron–motor neuron co-cultures were prepared according to conventional procedures\(^4\). Dishes of sensory neuron–motor neuron co-cultures were plated with 5–10 sensory neurons. Dishes of sensory neuron–motor neuron co-cultures were plated with a single sensory neuron and a single L7 motor neuron. Both sensory neuron cultures and sensory neuron–motor neuron co-cultures were allowed to grow for 5 d at 18 °C, and the growth medium was replaced before treatments and recordings with a solution of 50% L15 and 50% artificial seawater (ASW; 450 mM NaCl, 10 mM KCl, 11 mM CaCl\(_2\), 29 mM MgCl\(_2\), and 10 mM HEPES at pH 7.6). LTF was induced by repeated 5-HT treatment. Five 5-min pulses of either vehicle (L15:ASW) or 50 μM 5-HT (Sigma) were applied to the bath with ISIs as described (see Fig. 1b).\(^5\)

Stimulation of presynaptic sensory neurons was performed extracellularly using a blunt patch electrode filled with L15:ASW. Intracellular recordings from motor neurons were made with 10–20 MΩ sharp electrodes filled with 3 M potassium acetate connected to an Axoclamp 2-B amplifier (Molecular Devices)\(^6\). Data acquisition and analyses of resting potential, input resistance, and amplitude of EPSPs were performed with pCLAMP 8 software (Molecular Devices). Motor neurons were current clamped at −90 mV before measurement of EPSPs. Pre-treatment measurements of EPSP amplitude varied from 5 to 45 mV. Cultures were excluded from further use if EPSPs were less than 5 mV or were large enough to trigger an action potential. Motor neurons that had initial resting potentials more positive than −30 mV or input resistances less than 10 MΩ were also excluded from the analyses. Immediately after the end of treatment with 5-HT, cultures were returned to culture medium. At 1, 2 and 5 d after treatment, EPSP amplitudes were assessed in the same manner as the baseline measurements. In cases in which the post-test EPSP amplitude was accompanied by an action potential, the EPSP amplitude was assigned a value of 45 mV because the largest synaptic potential obtained in our culture system never exceeded that amplitude. Statistical analyses (one-way ANOVA) indicated that the pretest scores for each group were not significantly different (\(F_{5,26} = 0.25\)). Thus, for statistical analysis, the amplitudes of the EPSPs at 1, 2 and 5 d after treatment were normalized to the EPSPs measured before treatment (that is, post/pre).

CREB1 phosphorylation. For immunofluorescence analysis, sensory neuron cultures were treated with five 5-min pulses of 50 μM 5-HT. The intervals between the start of the 5-HT pulses (that is, the ISIs) were either 20 min (standard protocol; Fig. 1b) or 10, 10, 5 and 30 min (enhanced protocol; Fig. 1c). A separate group of control cultures were not treated with 5-HT, but were treated with vehicle alone (L15:ASW) with an ISI of 20 min. Immediately or 18 h after the end of treatment, cells were fixed in a solution of 4% paraformaldehyde (wt/vol) in phosphate-buffered saline containing 30% sucrose (wt/vol) and blocked for 30 min at 20–22 °C in Superblock blocking buffer (Pierce) with 0.2% Triton X-100 and 3% normal goat serum (vol/vol). Subsequently, fixed cells were incubated overnight at 4 °C with antibody to pCREB1 (1:500), which specifically recognizes the phosphorylated form of *Aplysia* CREB1 protein\(^7\), followed by incubation with secondary antibody (goat antibody to rabbit IgG conjugated to Cy-3; I:200) and Jackson ImmunoResearch) for 1 h at 20–22 °C. Images were obtained with a Zeiss LSM510 confocal microscope using a 63× oil immersion lens. A 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 nuclear fluorescence intensity with the MetaMorph Offline software (Universal Imaging). We analyzed five to ten neurons on each coverslip and averaged the measurements from neurons on the same coverslip. The levels of phosphorylated CREB1 in the standard and enhanced groups were normalized to the vehicle controls (Fig. 3b). The number of samples (n) reported in the text indicates the number of dishes. This experiment was done in a blind manner because different investigators carried out 5-HT treatments and immunofluorescence staining and image analysis.

Behavioral training. *Aplysia californica* (100–150 g) were obtained from Alacrity Marine Biological and Marinus. Animals were prepared for LTS training as described previously.\(^8\) Test stimuli were delivered through a pair of silver-wire electrodes implanted in each side of the tail, and siphon withdrawals were elicited by a brief electrical stimulus delivered to the tail. Prior to testing, a stimulus threshold (that is, the minimum amount of stimulating current necessary to elicit a withdrawal response) was determined for each side (contra- and ipsilateral to the training site; see below) of each animal (animals in the standard and enhanced groups). Statistical analyses (one-way ANOVA) indicated that the intensities of the threshold stimuli were not significantly different among the four measurements (\(F_{3,56} = 0.46\)). The overall average for the threshold stimulus was 1.97 ± 0.13 mA (mean ± s.e.m.). The intensity of subsequent test stimuli was set to 170% of the previously determined threshold for each side of each animal. Following a pre-training assessment of the strength of the reflex elicited by stimuli to the left and right side of the animal, five trains (1 Hz, 10 s) of sensitizing stimuli were delivered at the same intertrial intervals as used for the electrophysiological experiments on LTF: Sensitizing stimuli were applied to the lateral body wall of one randomly chosen side of the animal via a hand-held electrode. The left side was used for training in roughly the same number of experiments as the right. Because sensitization is lateralized,\(^9\) the contralateral side of each animal was used as a control. The post-training assessment was performed 1 and 5 d after training. The duration of siphon withdrawal was measured in response to each of five test stimuli. Data were excluded if the animals failed to respond to at least three of the five test stimuli because failed responses suggested nonfunctioning stimulating electrodes. The average of positive responses before and after training were calculated, and the averaged response durations 1 and 5 d after training were expressed as the ratio ‘post/pre’ (see Fig. 4). Statistical analyses (one-way ANOVA) indicated that the ipsi- and contralateral pretest responses for each group were not significantly different (\(F_{5,30} = 0.14\)). The average pretest responses (mean ± s.e.m.) for the contra- and ipsilateral sides in the standard group were 4.4 ± 0.9 and 3.9 ± 0.6 s, respectively. The average pretest responses for the contra- and ipsilateral sides in the enhanced group were 3.8 ± 0.6 s and 3.9 ± 0.5 s, respectively. Statistical analyses (two-sample t test) also indicated that the post-test contralateral responses (i.e., control responses) were not significantly different between the standard and enhanced groups one day after training (\(t_{20} = 1.32\)) or 5 d after training (\(t_{14} = 0.01\)). Thus, the contralateral responses for the standard and enhanced groups were combined into one control group for 1 d and another control group for 5 d after training (Fig. 4). In all experiments, different investigators carried out testing and training, and the investigator testing the animals was unaware of their prior treatment. All behavioral experiments were conducted at 15 °C.

Statistical analysis. All statistical analyses were performed using SigmaPlot (version 11) software (Systat Software). The specific types of statistical tests, the n for each analysis, P values and other relevant statistical values (for example, means, s.e.m., F, q and t values) are given at the appropriate places throughout the text.

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