cAMP Evokes Long-Term Facilitation in *Aplysia* Sensory Neurons That Requires New Protein Synthesis

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Behavioral sensitization leads to both short- and long-term enhancement of synaptic transmission between the sensory and motor neurons of the gill-withdrawal reflex in *Aplysia*. Serotonin (5-HT), a transmitter important for short-term sensitization, can evoke long-term enhancement of synaptic strength detected 1 day later. Because 5-HT mediates short-term facilitation through adenosine 3′,5′-monophosphate (cAMP)–dependent protein phosphorylation, the role of cAMP in the long-term modulation of this identified synapse was examined. Like 5-HT, cAMP can also evoke long-term facilitation lasting 24 hours. Unlike the short-term change, the long-lasting change is blocked by anisomycin, a reversible inhibitor of protein synthesis, and therefore must involve the synthesis of gene products not required for the short-term change.

THE GILL- AND SIPHON-WITHDRAWAL reflex in *Aplysia* can undergo short-term sensitization, lasting minutes to hours, and long-lasting sensitization, lasting days to weeks (*1*). Cellular studies of sensitization indicate that both the short- and the long-term changes involve heterosynaptic enhancement of transmitter release from the siphon sensory neurons onto gill and siphon motor cells (*2*). In both cases, this presynaptic facilitation of transmitter release is evoked by a common extracellular signal, 5-HT (*3, 4*), and is associated with a reduction in the 5-HT–sensitive K^+ current of the sensory neurons (*5, 6*) and an increase in neuron excitability (*7, 8*). The cellular changes associated with short-term sensitization involve a transmitter-mediated increase in cAMP within sensory cells, and a corresponding increase in cAMP-dependent phosphorylation of substrate proteins (*9*). Because both short- and long-term changes are evoked by 5-HT, and because they share several common effector features, they could also share the same intracellular second messenger signaling system. Alternatively, because long-term sensitization differs from short-term sensitization in requiring new protein synthesis (*10*) and involving new synaptic growth (*11*), the long-term change might require parallel activation of other second messenger systems in the sensory neurons.

To explore these alternatives, we have examined whether cAMP can produce long-term enhancement in the excitatory postsynaptic potential (EPSP) of the monosynaptic sensory-to-motor component of the gill-withdrawal reflex in dissociated cell culture (Fig. 1A). After 5 days in culture, the amplitude of the EPSP evoked in L_7 is stable. This stable EPSP can, however, undergo short- and long-term enhancement. Thus, whereas a single application of 5-HT can evoke facilitation lasting only minutes, four or five repeated 5-min applications over a total of 1.5 hours result in a facilitation that persists more than 1 day (*4*). In culture, a single 15-min bath application of the cAMP analog 8-benzylthio adenosine 3′,5′ monophosphate, in the presence of isobutyl methylxanthine (IBMX), a phosphodiesterase inhibitor (*12*), produces a transient facilitation (Table 1). Because repeated applications of 5-HT over 1.5 hours would increase cAMP repeatedly (*9*), we examined whether exposures to the cAMP analog for a similar period (1.5 to 2 hours) would lead to long-term facilitation.

We measured the amplitude of the EPSP between each sensory and motor cell (Fig. 1B, 0 hour) and perfused the cells with the cAMP analog and IBMX for 1.75 hours, followed by a 15-min rinse (*13*). Twenty-four hours later, we reexamined the connections between the cells (Fig. 1B, 24 hours) and found that treatment with the cAMP analog plus IBMX evoked a significant increase in the EPSP amplitudes of 52 ± 5% (mean ± SEM). By contrast, untreated control cultures or cultures exposed to IBMX alone showed a small decrease of −8 ± 4% and −9 ± 8%, respectively, in their synaptic strength. This decrease was not significantly different from the initial levels (Fig. 1C). For all conditions, there was no significant change in the resting potential, input resistance, or action potential of the motor cell. This long-term change evoked by cAMP appears to be specific. Incubation for equal time periods with activators of other second messenger systems failed to produce long-term facilitation. Thus, cultures exposed to 100 n*M* phorbol esters (phorbol 12,13-dibutyrate), which can facilitate sensorimotor connections in vivo and in vitro (*14*), showed no significant change in their EPSP amplitudes when retested 24 hours later (−2 ± 11%, $n = 6$) compared to the untreated control cultures (7 ± 12%, $n = 5$). In contrast, incubations with 10 μ*M* arachidonic acid, which gives rise to lipoxygenase metabolites that serve as second messengers

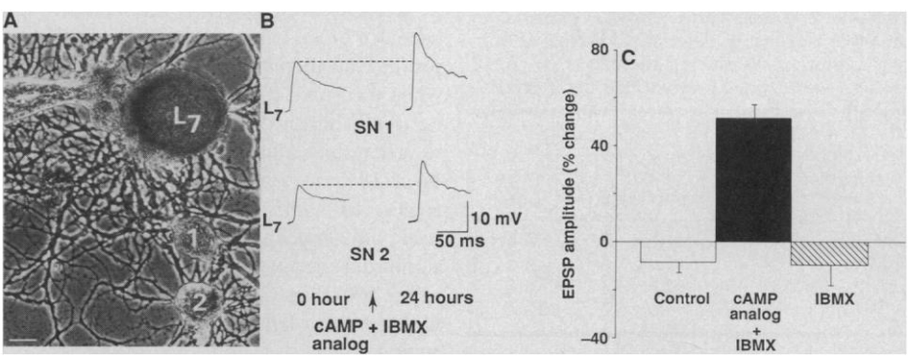

Fig. 1. Long-term facilitation of the connections between sensory and motor cells produced by the cAMP analog (8-benzylthio cAMP) and IBMX. (**A**) Phase light micrograph of a culture with two sensory neurons (SN 1 and SN 2) and motor cell L_7 taken after the second recording session, 24 hours after incubation with cAMP analog plus IBMX. Bar, 50 μm. (**B**) Analog plus IBMX enhances EPSP amplitude recorded 24 hours after incubation. The EPSPs were recorded in the motor cell in (A) upon stimulation of sensory neuron 1 (SN 1) and 2 (SN 2) with an extracellular electrode before (0 hour) and 24 hours after incubation with analog and IBMX. (**C**) Summary of cAMP analog-induced long-term facilitation. The height of each bar is the mean ± SEM of the percent change in the amplitude of the EPSP recorded at 0 hour compared to the EPSP of the same connection recorded 24 hours later. The results from each culture (consisting of one L_7 cell and one to three sensory neurons) were averaged and treated as a single sample. Nine connections were examined for each experimental condition. In cases where more than one connection was present in a single culture [as in (B)], the percent change was calculated as the mean change for all connections. An overall analysis of variance indicated a difference with treatment ($F = 37.05$; df = 2,12; $P < 0.001$). A comparison of the means (Dunnett's test) indicated that cAMP analog plus IBMX significantly increased EPSP amplitude compared to the control ($t = 7.39$, $P < 0.01$) or IBMX ($t = 7.51$, $P < 0.01$), whereas the control and IBMX groups did not differ from each other; $n = 5$ for each experimental condition.

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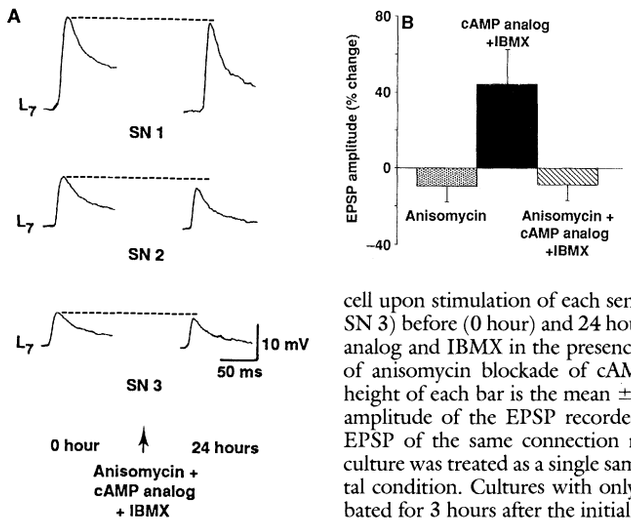

Fig. 2. Suppression of protein synthesis during and immediately after incubation with cAMP analog and IBMX blocks long-term facilitation. The protocol used was the same as that described in Fig. 1 and the text. (**A**) Anisomycin blocks cAMP long-term facilitation. The EPSPs were recorded from a single motor cell upon stimulation of each sensory neuron (SN 1, SN 2, and SN 3) before (0 hour) and 24 hours after incubation with cAMP analog and IBMX in the presence of anisomycin. (**B**) Summary of anisomycin blockade of cAMP long-term facilitation. The height of each bar is the mean ± SEM of the percent change in amplitude of the EPSP recorded at 0 hour, compared to the EPSP of the same connection recorded 24 hours later. Each culture was treated as a single sample; $n = 5$ for each experimental condition. Cultures with only anisomycin added were incubated for 3 hours after the initial recording (0 hour). An overall analysis of variance indicated a difference with treatment ($F = 6.44$; df = 2,12; $P < 0.025$). A comparison of the means (Dunnett's test) indicated that analog plus IBMX increased significantly the EPSP compared to the other treatments ($t = 3.15$, $P < 0.01$ for anisomycin; $t = 3.06$, $P < 0.01$ for anisomycin plus analog and IBMX), whereas anisomycin alone and analog plus IBMX in the presence of anisomycin did not differ from each other. Cultures ($n = 3$) exposed to anisomycin for 2 hours were capable of undergoing analog-induced, short-term changes.

Table 1. Short-lasting facilitation of sensorimotor connections produced by cAMP analog plus IBMX. The values (mean ± SEM) were measured on cells as in Figs. 1 and 2 and as described in (*13*). After recording the amplitude and shape of the initial EPSP, the cells were perfused for 15 min with their respective drug treatments and the connections reexamined after 3 min. The presence of cAMP analog plus IBMX significantly increased both the amplitude ($F = 4.49$, df = 2,12, $P < 0.05$; Dunnett's test, $t = 2.40$, $P < 0.05$ versus control, and $t = 2.76$, $P < 0.05$ versus IBMX) and the time-to-peak phase of the EPSP ($F = 27.55$, df = 2,12, $P < 0.001$; Dunnett's test, $t = 6.35$, $P < 0.01$ versus control, and $t = 6.49$, $P < 0.01$ versus IBMX) compared to the other treatments. After an additional 15 min of washout (+ Washout), the effects of cAMP analog were reversed nearly to the initial levels.

Drug treatment	n	% Change	
		EPSP amplitude	Time-to-peak
Control	5	−10 ± 6	−3 ± 5
IBMX	5	−12 ± 6	−4 ± 3
cAMP + IBMX	5	+22 ± 13	+62 ± 11
+ Washout	5	+6 ± 7	+26 ± 5

in mediating short-term presynaptic inhibition of this synapse (*15*), produced a significant long-term decrease in the amplitude of the EPSP ($n = 5$) by −35 ± 9% (*16*).

Three features of long-term sensitization are blocked by inhibitors of protein or RNA synthesis when applied either during behavioral training or in culture during the period of 5-HT application: (i) long-term behavioral sensitization of the reflex (*10*), (ii) long-term facilitation of the sensorimotor connections (*4*), and (iii) long-term increase in the excitability of the sensory neurons (*8*). By contrast, the parallel short-term behavioral and cellular processes are not affected by these inhibitors. We therefore examined whether the long-term facilitation induced by cAMP is similarly blocked by anisomycin, a reversible inhibitor of protein synthesis (Fig. 2). We repeated the previous experiment and treated the cultures for 1.75 hours with the cAMP analog plus IBMX. In addition, we exposed these cultures to 10 μM anisomycin for 3 hours (from 15 to 30 min before until 45 to 60 min after exposure to the cAMP analog plus IBMX). In the presence of anisomycin, the analog failed to produce an increase in the EPSP amplitude when measured 24 hours later (Fig. 2A, 24 hours). Whereas the analog increased synaptic strength in a second group of cultures by 44 ± 18%, anisomycin reduced the effect of analog to control range (−8 ± 7%). By itself, anisomycin produced a small but not significant decrease in EPSP amplitude of −9 ± 8% (Fig. 2B) and did not interfere with the short-lasting changes evoked by the analog.

Our findings indicate that the monosynaptic connection between sensory and motor cells of the *Aplysia* gill-withdrawal reflex—a site of plasticity that contributes to behavioral sensitization—can show both short- and long-term facilitation with application of cAMP analog. This suggests that up to this point in the chain of signal transduction, the short- and long-term forms of plasticity are in series with one another. This may explain why several of the steps initiated by cAMP in the short-term process (*9*), such as enhancement of transmitter release, depression of K^+ currents, and increase in excitability, also participate in the long-term process. However, it is still not known whether 5-HT or other facilitating transmitters induce all of their long-term effects through cAMP, or whether additional second messengers are involved. The significant role of cAMP in both short- and long-term sensitization in *Aplysia* is also supported by the parallel biochemical studies of Greenberg *et al.* (*17*) and by the biophysical studies on the tail withdrawal reflex of *Aplysia* (*18*). As described in (*19*), intracellular injection of cAMP into individual sensory neurons can evoke long-term (24 hours) changes in membrane currents similar to those observed 24 hours after long-term sensitization training of the tail-withdrawal reflex (*6*).

As for long-term facilitation and the excitability change produced by repeated applications of 5-HT (*4, 8*), the long-term change evoked by cAMP is blocked selectively by inhibitors of protein synthesis applied during the acquisition phase. Thus, unlike the short-term process that involves posttranslational modifications of preexisting molecules (*9, 20*) and does not require new macromolecular synthesis (*21*), long-term facilitation requires new macromolecular synthesis. Because in some systems cAMP can regulate gene expression (*22*), the expression of long-term facilitation may represent a cAMP-mediated inductive process. Recent results indicate that long-term sensitization of the reflex leads to an alteration in the level of synthesis of certain specific proteins (*10*). One now needs to determine the mechanism whereby cAMP leads to these changes in protein synthesis, and how, in turn, the alterations in protein synthesis affect the cellular events associated with long-term sensitization.

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12. For all experiments, we used $10^{-4}M$ cAMP analog (ICN), plus $5 \times 10^{-4}M$ IBMX (Sigma). By itself, this concentration of analog failed to produce either short- ($n = 3$) or long-term ($n = 3$) facilitation. Higher concentrations of cAMP analog could not be used because they caused the normally silent sensory neurons to become spontaneously active and fire action potentials.
13. Cultures were prepared by methods in (*3*), (*4*), and (*8*). The motor cell was impaled with an intracellular electrode filled with 1.5*M* KCl and held with hyperpolarizing current injection at 30 mV below the resting potential. The sensory neurons were stimulated with brief (0.2 to 0.4 ms) depolarizing pulses with an extracellular electrode filled with perfusion medium (equal volumes of Leibovitz's L-15 culture medium adjusted for marine saline conditions and artificial seawater plus *Aplysia* hemolymph to a final concentration of 2.5%). After the initial recordings of the EPSP, the cells were perfused with medium containing control or experimental drug treatments and maintained at 19°C. The cells were then rinsed in perfusion medium, returned to normal culture medium consisting of equal volumes of *Aplysia* hemolymph and L-15 medium, and placed back into the incubator at 18°C. The cells were reexamined 24 hours later with the same procedures.
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Tandem Array of Human Visual Pigment Genes at Xq28

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Unequal crossing-over within a head-to-tail tandem array of the homologous red and green visual pigment genes has been proposed to explain the observed variation in green-pigment gene number among individuals and the prevalence of red-green fusion genes among color-blind subjects. This model was tested by probing the structure of the red and green pigment loci with long-range physical mapping techniques. The loci were found to constitute a gene array with an approximately 39-kilobase repeat length. The position of the red pigment gene at the 5′ edge of the array explains its lack of variation in copy number. Restriction maps of the array in four individuals who differ in gene number are consistent with a head-to-tail configuration of the genes. These results provide physical evidence in support of the model and help to explain the high incidence of color blindness in the human population.

COLOR BLINDNESS IS A COMMON trait in humans. Approximately 1 in 12 Caucasian males and 1 in 200 females are color-blind (*1*). The vast majority of color blindness is the result of alterations in the genes encoding the red and green visual pigments located at the end of the long arm of the X chromosome (*2*). The physical distance between these genes is not known, but the fact that they are 98% identical in DNA sequence suggests they arose by an evolutionarily recent duplication event. Normal individuals possess from one to three green pigment genes but show no variation in the number of red pigment genes, which is always one (*3*). Examination of the structure of these loci in individuals with red or green color blindness revealed rearrangements of at least one of the genes in 24 of 25 cases (*2*). All of these rearrangements appeared to arise from homologous recombination, resulting in either a complete gene deletion or the production of fusion genes. This situation contrasts with that of other loci such as clotting factor VIII, ornithine transcarbamylase, and hypoxanthine-guanine phosphoribosyltransferase in which 2, 7, and 18%, respectively, of observed mutations are rearrangements and most or all of these are intragenic deletions (*4–6*).

A model for the arrangement of the red and green visual pigment genes has been proposed to explain these observations (*3*). It specifies a head-to-tail tandem array of genes composed of the red pigment gene at the 5′ end with a variable number of green pigment genes 3′ to it. Homologous but unequal crossing-over in the array can generate the variation in green pigment gene number observed in normal subjects as well as the gene fusions and deletions observed in color-blind individuals. The fact that an entire red pigment gene is never duplicated or deleted is explained in this model by its position at the 5′ end of the array, abutting unique sequences.

The model makes several specific predictions about the structure of the visual pigment loci as a function of the number of genes possessed by an individual (see below). These predictions could be experimentally tested by DNA physical mapping methods if the red and green pigment genes were sufficiently close together. To assess the distance between the two genes, very high molecular weight DNA prepared from the peripheral blood leukocytes of a normal male with one red and one green pigment gene was digested with restriction enzymes that cleave infrequently in the human genome. The digested DNA was fractionated on the basis of size by means of alternating contour-clamped homogeneous electric field (CHEF) gel electrophoresis, transferred to a membrane, and hybridized with a complementary DNA (cDNA) probe for the red pigment gene (Fig. 1) (*7, 8*). Because of the high degree of similarity between the genes, this probe detects the green pigment gene as well.

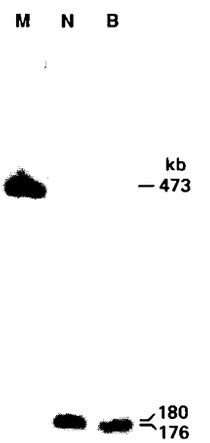

Fig. 1. Physical linkage of the red and green visual pigment genes. Very high molecular weight DNA from an individual with one red and one green pigment gene (1:1) was prepared and digested as described (*8*) with Mlu (M), Nru I (N), and BssH II (B). Samples were fractionated by alternating CHEF gel electrophoresis (*7*) (200 volts, 23 hours, 1% agarose, 30-second switching interval, 9°C). The DNA was then transferred to Genetran (Plasco) and hybridized with a full-length cDNA encoding the red visual pigment (*3*).

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