Serotonin Induces Temporally and Mechanistically Distinct Phases of Persistent PKA Activity in Aplysia Sensory Neurons

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Summary

The cAMP signaling cascade has been implicated in several stages of memory formation. We have examined activation of this cascade by serotonin (5-HT) in the sensory neurons of Aplysia. We find that different patterns of 5-HT exposure induce three distinct modes of PKA activation. First, a single 5 min pulse induces transient (5 min) PKA activation that requires neither transcription nor translation. Second, 4-5 pulses induce intermediate-term persistent activation (3 hr duration) that requires translation but not transcription. Third, 5 pulses of 5-HT, as well as continuous (90 min) exposure, induce long-term persistent activation 20 hr later, which requires both transcription and translation. Thus, in the sensory neurons, different patterns of 5-HT give rise to three independent phases of PKA activation that differ in their induction requirements, their temporal profiles, and their molecular mechanisms.

Introduction

Memories can exist in a variety of temporal domains, ranging from the short-term encoding of events lasting seconds to minutes to long-term storage of information lasting days, weeks, and, in the limit, a lifetime. Considerable experimental effort on the behavioral level has been focused on identifying the temporal dynamics of different phases of memory in a wide variety of systems, from invertebrate animals such as the honeybee (Hammer and Menzel, 1995), Drosophila (DeZazzo and Tully, 1995), and Aplysia (Carew et al., 1972; Byrne, 1987; Carew, 1987; Hawkins et al., 1987) to higher animals (McGaughr, 1966) including nonhuman primates (Goldman-Rakic, 1992) and humans (Atkinson and Shiffrin, 1968). Two seminal advances in the field of memory research have provided critical insights into the mechanisms involved in the transition from short-term memory to long-term memory. First, it has long been appreciated that the formation of long-term memory, or its cellular analog, long-term synaptic facilitation, requires de novo gene expression (Agranoff et al., 1966; Barondes and Cohen, 1968; Davis and Squire, 1984; Montarolo et al., 1986; Nguyen et al., 1994; Tully et al., 1994). Second, it is also now clear that the second messenger cAMP plays a pivotal role in the induction of the neural substrates of long-term memory in a number of systems, including Aplysia (Dash et al., 1990; Ghirardi et al., 1992; Alberini et al., 1994; Bartsch et al., 1995; Drosophila (Drain et al., 1991; Davis et al., 1995; DeZazzo and Tully, 1995), honeybee (Hildebrandt and Müller, 1995a, 1995b; Hammer and Menzel, 1995), and the hippocampus (Frey et al., 1993; Huang et al., 1994; Huang et al., 1995; Impey et al., 1996). Taken together, these two advances suggested a role for cAMP-dependent gene expression in the induction of long-term memory. Recently, it has become clear that this is indeed the case. Specifically, a subset of genes from the cAMP response element binding protein (CREB) family, which are known to mediate cAMP-dependent transcription, have been directly implicated in long-term memory formation.

Experiments in Aplysia examining long-term synaptic facilitation (LTF) in cultured sensory and motor neurons have shown that cAMP-dependent transcription via CREB plays a critical role in the induction of LTF (Dash et al., 1990; Alberini et al., 1994; Bartsch et al., 1995; Martin et al., 1997; see also Carew, 1996). Work in Drosophila has shown that CREB plays a primary role in the induction of long-term memory for odors in a classical conditioning paradigm (Tully et al., 1994; Yin et al., 1994). Particularly compelling, in both of these systems, is the demonstration that LTF in Aplysia and long-term memory in Drosophila are not only impaired by interfering with CREB activation but are enhanced by enhancing CREB function (Bartsch et al., 1995; Yin et al., 1995; see also Carew, 1996). In addition to having a role in these invertebrate systems, CREB has been implicated in memory formation in mice (Bourchuladze et al., 1994) and in the induction of long-term potentiation (LTP) in hippocampus (Impey et al., 1996).

Given the central involvement of the cAMP signaling cascade in memory formation, we were interested in examining in detail the time course and mechanisms underlying the activation of the cAMP signaling that might contribute to different phases of memory processing. The tail withdrawal reflex in Aplysia provides an experimental system that is particularly well suited for this type of analysis. Monosynaptic connections between identified tail sensory neurons in the pleural ganglion and tail motor neurons in the pedal ganglion are known to exhibit both short-term facilitation (STF) in response to a single noxious tail stimulus and LTF in response to repeated tail stimuli (Walters et al., 1983; Buonomano and Byrne, 1990; Mercer et al., 1991). Moreover, both the short-term and long-term effects of tail shock are mimicked by single or repeated pulses of the biogenic amine serotonin (5-HT) to the CNS (Walters et al., 1983; Mercer et al., 1991; Emptage and Carew, 1993; MAuelshagen et al., 1996; Zhang et al., 1997). Using this system, MAuelshagen and colleagues (1996) showed that STF induced by repeated pulses of 5-HT decays to baseline several hours before the expression of LTF.
Aplysia expression of LTF. control for variability between sensory cells from dif- cayed to baseline several hours before the ultimate ex- for different lengths of time prior to rapid freezing. To Mauelshagen et al. (1996) found that STF and ITF de- a dose±response study by incubating dissociated cells that ITF depended exclusively on translation but not of 5-HT and different durations of 5-HT application on transcrip- tion of new protein. Finally, as mentioned above, PKA activity in intact sensory neurons, we carried out cultured

Moreover, temporally spaced exposure of 5-HT 5-HT concentration (50

M) led to a reduction in PKA activity, as is common for dose±response curves. As shown in Table 1, by far the highest incorporation into I-1 is caused by stimulation of homogenates of sensory neurons with Br2CAMP. The 32P incorporation into I-1 in the presence of activators of kinases other than PKA does not differ from that of the control. Thus, the heat-stable protein I-1 is a suitable specific substrate of PKA in Aplysia.

By examining the effects of different numbers of pulses of 5-HT, Mauelshagen et al. (1996) found that 1 to 4 pulses induce a rapidly decaying STF (lasting 10±15 min) but an additional fifth pulse induced a pro- longed intermediate-term facilitation (ITF) that lasts up to 120 min. This observation confirmed results obtained by Ghirardi et al. (1995), who first described ITF in cul- tured Aplysia sensory neurons. Importantly, they found that ITF depended exclusively on translation but not transcription of new protein. Finally, as mentioned above, Mauelshagen et al. (1996) found that STF and ITF de- cayed to baseline several hours before the ultimate expression of LTF.

The work described above shows that, in the tail sen- sory±motor system of Aplysia, repeated pulses of 5-HT induce three distinct phases of synaptic facilitation: STF (lasting 10-15 min), ITF (lasting 1-3 hr), and LTF (lasting longer than 24 hr). Since, as we have discussed, cAMP is known to be involved presynaptically in facilitation of Aplysia sensory-motor synapses (reviewed by Byrne and Kandel, 1996), in the present paper we asked whether distinct temporal phases of activation of the cAMP cascade are induced by 5-HT in the tail sensory neurons. To examine this question, we measured directly changes in PKA activity induced by 5-HT stimulation of sensory neuron cell bodies following 5-HT treatment in the intact CNS using the method developed by Hildebrandt and Müller (1995a, 1995b).

Table 1. Specificity of Protein I-1 Phosphorylation by Homogenates of Sensory Neurons

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Relative 32P Incorporation into I-1</th>
</tr>
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<tbody>
<tr>
<td>CONTROL (ASW)</td>
<td>7% ± 4 (6)</td>
</tr>
<tr>
<td>CONTROL (EGTA, 5 mM)</td>
<td>6% ± 5 (5)</td>
</tr>
<tr>
<td>Ca2+ (2 mM)</td>
<td>8% ± 10 (5)</td>
</tr>
<tr>
<td>PKA (Br2CAMP, 10 μM)</td>
<td>100% ± 11 (8)*</td>
</tr>
<tr>
<td>PKC (Ca2+/diacyl glycerol)</td>
<td>11% ± 7 (9)</td>
</tr>
<tr>
<td>CAMKII (Ca2+/calmodulin)</td>
<td>10% ± 8 (6)</td>
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</tbody>
</table>

Homogenized sensory neurons were subjected to a kinase assay (Hildebrandt and Müller, 1995a, 1995b) in the presence or absence of the indicated activators of protein kinases. After 30 s incubation, the reaction was terminated by the addition of SDS sample buffer, and the samples were subjected to SDS-PAGE. The 32P incorporation into the externally added substrate protein I-1 was determined. Values were normalized with respect to the 32P incorporation into I-1 by stimulation with Br2CAMP. Means ± SEM are presented. The number of measurements is shown in parenthesis. Only 32P incorporation into I-1 induced by Br2CAMP significantly differs from control (p < 0.01).

Furthermore, by examining the effects of different num- bers of pulses of 5-HT, Mauelshagen et al. (1996) found that 1 to 4 pulses induce a rapidly decaying STF (lasting 10-15 min) but an additional fifth pulse induced a pro-longed intermediate-term facilitation (ITF) that lasts up to 120 min. This observation confirmed results obtained by Ghirardi et al. (1995), who first described ITF in cul-tured Aplysia sensory neurons. Importantly, they found that ITF depended exclusively on translation but not transcription of new protein. Finally, as mentioned above, Mauelshagen et al. (1996) found that STF and ITF de-cayed to baseline several hours before the ultimate expression of LTF.

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We report here that that temporally spaced pulses of 5-HT induce three mechanistically distinct phases of PKA activation in the sensory neurons that are dynamically quite similar to STF, ITF, and LTF (Mauelshagen et al., 1996). Moreover, temporally spaced exposure of 5-HT has different inductive properties than massed exposure. Thus, the transition from one phase of PKA activation to another depends upon the number and pattern of 5-HT exposure, which induce modes of activation that have separate and distinct molecular requirements.

**Results**

5-HT Induces PKA Activation in the Sensory Neurons

Although it has been convincingly demonstrated that 5-HT induces an increase in cAMP in the sensory neurons of Aplysia (Bernier et al., 1982; Greenberg et al., 1987; Bacskai et al., 1993; see also Byrne and Kandel, 1996), the temporal dynamics of PKA activation following different patterns of 5-HT stimulation have not been analyzed. To examine this question, we determined changes in PKA activity induced by 5-HT stimulation of Aplysia sensory neurons using a technique originally optimized for insects (Hildebrandt and Müller, 1995a, 1995b). To apply this technique, we first had to verify the specificity of this method and adapt it for the analysis of Aplysia neurons. The technique is comprised of two steps: (1) the preservation of stimulus-induced changes in PKA activity by rapid freezing of the sensory neurons, and (2) the determination of PKA activity by a fast and specific in vitro kinase assay. First, we confirmed that the specific PKA substrate used in insects, phosphatase inhibitor 1 (I-1), is also specifically phosphorylated by Aplysia PKA. As shown in Table 1, by far the highest incorporation into I-1 is caused by stimulation of homogenates of sensory neurons with Br2CAMP. The 32P incorporation into I-1 in the presence of activators of kinases other than PKA does not differ from that of the control. Thus, the heat-stable protein I-1 is a suitable specific substrate of PKA in Aplysia.

To examine the effects of increasing concentrations of 5-HT and different durations of 5-HT application on PKA activity in intact sensory neurons, we carried out a dose–response study by incubating dissociated cells for different lengths of time prior to rapid freezing. To control for variability between sensory cells from different ganglia, each cluster was dissociated and divided into four fractions. This procedure allowed us to determine for each cluster (1) the basal PKA activity (cell fraction incubated in artificial seawater [ASW]), (2) the maximal PKA activity (cell fraction stimulated with Br2CAMP), (3) PKA activity during 5-HT application, and (4) PKA activity at different time points following 5-HT. Figure 1A shows a representative autoradiograph with the phosphorylation pattern derived from the differently stimulated fractions of a sensory cell cluster. The differences in 32P incorporation into the PKA substrate I-1 (arrow) are unambiguous. In all following figures, changes in PKA activity between the basal and the maximal values of each cluster (indicated by dashed lines) were normalized to 0 and 1, respectively.

The effect of increasing concentrations of 5-HT on PKA activity in the sensory neurons is shown in Figure 1B. A 20% increase in PKA activity was already induced by the incubation of the neurons in 0.2 μM 5-HT (3 min). The maximal increase in 5-HT-induced PKA activity was achieved by concentrations of 2-20 μM, while a higher 5-HT concentration (50 μM) led to a reduction in PKA activity, as is common for dose–response curves. As shown in Figure 1C, PKA activity declines significantly during incubation of the sensory neurons with 50 μM 5-HT. At lower concentrations of 5-HT (e.g., 10 μM), no such reduction of PKA activity during the 5 min pulse.
Figure 1. Effect of 5-HT on the PKA Activity in Aplysia Sensory Neurons

Dissociated sensory neurons were stimulated either by ASW alone (control) or by ASW containing BrCAMP (10 μM) or 5-HT (10 μM).

(A) A representative autoradiograph illustrates the distinct \(^3^P\) incorporation into the PKA substrate I-1 (arrow). Other bands reflect incorporation of \(^3^P\) into intrinsic proteins induced by the activity of endogenous kinases.

(B) Effects of different 5-HT concentrations (3 min exposure) on PKA activity. In this and other figures, values represent the relative change in PKA activity calculated with respect to the basal and maximal activities (normalized as 0.0 and 1.0, respectively; dashed lines), which correspond to the \(^3^P\) incorporation induced either by ASW- (control) or by BrCAMP-stimulated sensory neurons (see [A]). Each data point represents the mean \pm SEM of at least 12 measurements, and each point significantly differs from ASW-stimulated control (basal activity) (p < 0.01).

(C) PKA activity determined at different time points after the application of either 10 μM or 50 μM 5-HT. Each point represents the mean \pm SEM of relative PKA activity of at least 12 measurements. While PKA activity induced by 10 μM 5-HT remains at a high level, PKA activity induced by 50 μM 5-HT significantly declines during 5-HT exposure (p < 0.05).

is observed. Based on these findings, we used a concentration of 10 μM 5-HT in all of the experiments that follow. This 5-HT concentration is commonly used to examine synaptic facilitation in the sensory neurons (Mercer et al., 1991; Emptage and Carew, 1993).

Temporal Dynamics of PKA Activation during Repeated Pulses of 5-HT

Previous studies examining synaptic facilitation in the sensory neurons have shown that a single 5-HT pulse (5 min) leads only to short-term effects, whereas repeated pulses of 5-HT, with interstimulus intervals of 15 min, induce long-term changes in the sensory neurons (Montarolo et al., 1986; Emptage and Carew, 1993; Ghirardi et al., 1995; Mauelshagen et al., 1996). In addition, PKA activation is required for long-term changes in sensory neurons (see Byrne and Kandel, 1996). These two observations led us to examine the modulation of PKA activity induced by 5 repeated pulses of 5-HT. Toward that end, we measured PKA activity in the sensory neurons at different times before and after the first, third, and fifth 5-HT pulses (Figure 2). To minimize the variability between ganglia from different animals, the two pleural-pedal ganglia from each animal were always treated in parallel; one as the experimental ganglion, the other as control (see Experimental Procedures). This procedure also permitted us to ensure that there was no change in either basal or maximal PKA activity.

5-HT was delivered either to intact (desheathed) pleural-pedal ganglia or to isolated sensory neuron somata, depending upon the phase of the experiment. Since the measurement of PKA activity required rapid freezing of the somata to arrest PKA-induced phosphorylation (see Experimental Procedures), in many cases the last 5-HT pulse in a series, or all test pulses of 5-HT (see below), required that this 5-HT pulse be delivered directly to the isolated somata in vitro. For example, for the determination of PKA activation induced by three pulses of 5-HT (separated by 15 min), the first and the second 5-HT pulses were delivered to the pleural-pedal ganglia, which were then dissected and divided into four fractions prior to delivery of the third 5-HT pulse in vitro. Correspondingly, for determining the effects of 5 pulses of 5-HT, the first 4 pulses were delivered to the desheathed ganglia, while the fifth pulse was delivered to isolated somata in vitro. Thus, with this procedure, the induction of changes in PKA activity may involve processes activated in the ganglion by 5-HT (in addition to...
somatic processes), whereas the expression of these changes at all time points following training can be attributed solely to somatic processes in the sensory neurons.

As shown in Figure 2, PKA activity after the first and the third 5-HT pulse was transient, returning to basal PKA activity levels about 5 min after the end of the 5-HT pulse. Interestingly, before the fifth 5-HT pulse, PKA activity was persistently elevated (0.42 ± 0.09) and was significantly different from basal PKA activity of the control side treated with ASW (p < 0.01). This persistent activity was thus induced by the fourth pulse of 5-HT. The fifth 5-HT pulse led to a further significant increase in PKA activity (0.72 ± 0.06), which remained at the elevated level (0.63 ± 0.09) for at least 20 min. Although, as described above, 5-HT could induce a difference in basal (persistent) activity between experimental and control ganglia, in no case did we detect a difference in the maximal PKA activity between groups. Thus, during repeated pulses of 5-HT, at least two modes of PKA activation can be distinguished: a transient activation of PKA during the first 5-HT pulses and a persistent increase in PKA activity that develops during the fourth and fifth 5-HT pulses.

Repeated Pulses of 5-HT Induce Intermediate-Term and Long-Term Changes in PKA Activity

Several previous studies have implicated the cAMP signaling cascade in different phases of synaptic plasticity induced by 5-HT in Aplysia sensory neurons (Ghirardi et al., 1995; Hegde et al., 1997). To further explore this effect, we measured PKA activity at 1, 3, and 20 hr after either 1 or 5 pulses of 5-HT. In addition, at each time point, a test pulse of 5-HT was also delivered to determine whether further activation of PKA was possible. In these experiments, 5-HT pulses (1 × 5-HT or 5 × 5-HT) were applied to the pleural-pedal ganglia. Ten minutes prior to the 1, 3, and 20 hr tests, the sensory neurons were dissected and divided into four fractions as described previously.

As shown in Figure 3, there was no persistent PKA activation 1, 3, and 20 hr after a single 5-HT pulse was applied to a pleural-pedal ganglion. However, the test pulse of 5-HT at each time point induced transient activation of PKA. A totally different picture emerged after multiple 5-HT pulses (Figure 3). One hour after the fifth 5-HT pulse, PKA activity was still elevated (0.49 ± 0.11) and was significantly different from the basal PKA activity of the control side (p < 0.01). The 5-HT test pulse at this time point induced no additional increase in PKA activity. Three hours after the fifth 5-HT pulse, persistent PKA activity of the sensory neurons had returned to the basal levels. At this time point, the 5-HT test pulse induced transient PKA activation. Interestingly, at 20 hr, after multiple 5-HT stimulation, persistent PKA activity (0.38 ± 0.1) was again evident in the sensory neurons. But, in contrast to the 1 hr test, the 5-HT test pulse induced a further transient increase in PKA activity. This pattern of results is consistent with observations of Ghirardi et al. (1995), who found that, during ITF, no further increase in synaptic facilitation was induced by a pulse of 5-HT, whereas during LTF further synaptic enhancement could be induced by transient exposure to 5-HT. The data shown in Figure 3 suggest the existence of both a persistent and a transiently regulated fraction of PKA activity. The long-term persistent fraction at 20 hr is very likely due to the persistent activation of PKA by the selective proteolysis of the regulatory subunit described by Schwartz and colleagues (Chain et al., 1995; Hegde et al., 1997). Moreover, since maximal PKA activity is not different between control and 5-HT-treated groups (data not shown), this indicates that the total pool of PKA activity is relatively constant. Thus, the increase in persistent activity at 20 hr reflects a shift in the fraction of that pool that is persistently activated and a corresponding decrease in the fraction that can be transiently activated by the test pulse.

Our results show that stimulation of sensory neurons by 5 repeated pulses of 5-HT induces at least three distinct modes in PKA activation: (1) transient activation after the first and third 5-HT pulses, (2) persistent elevation of PKA activity induced by repeated 5-HT pulses,
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Figure 4. Modulation of PKA Activity in the Sensory Neurons by Continuous Application of 5-HT

Although the PKA activity decreases significantly during the 90 min 5-HT pulse ($p < 0.01$), it remains significantly above baseline throughout 5-HT exposure. PKA activity decays to basal levels 5 min after the end of 5-HT exposure.

Massed Application of 5-HT Induces Only Long-Term Changes in PKA Activity

Although it has been demonstrated that prolonged, continuous application of 5-HT (massed application) can induce long-term synaptic facilitation (Emptage and Carew, 1993; Ghirardi et al., 1995; Zhang et al., 1997), it is not known whether such massed application of 5-HT induces distinct phases of synaptic facilitation similar to those induced by repeated application of 5-HT (spaced application) as described by Ghirardi et al. (1995) and Mauelshagen et al. (1996). To explore this question at the level of PKA activation, we asked whether a different pattern of 5-HT application (massed application for 90 min) induces a temporal pattern of PKA activation in the sensory neurons comparable to that induced by spaced application (Figures 2 and 3). Ninety minutes was chosen because it approximates the total (integrated) amount of PKA activation induced during 5 spaced pulses of 5-HT (Figures 2 and 3). Three time points were assessed during constant 5-HT exposure: 5 min, 35 min, and 90 min. For the 5 min test, 5-HT was delivered to isolated sensory neurons in vitro. For the 35 min and 90 min tests, 5-HT was applied to the pleural-pedal ganglia. A massed application of 5-HT (90 min) was delivered to isolated sensory neurons in vitro (as described above; see also Experimental Procedures).

As shown in Figure 4, PKA activity was increased during the entire 90 min of 5-HT application. Although the initially high PKA activity ($0.78 \pm 0.13$) slowly decreased until the end of the 90 min application (to $0.55 \pm 0.08$), it was still significantly elevated over basal levels of PKA activity. In contrast to the activation pattern induced by repeated 5-HT pulses (Figure 2), PKA activity returned to basal levels immediately after the end of a single 90 min 5-HT pulse. This observation was confirmed by the measurement of PKA activity at 1 hr after massed 5-HT application (Figure 5). These data show that massed application of 5-HT did not induce the intermediate-term (1 hr) phase of persistent PKA activation observed after spaced 5-HT applications (compare Figures 3 and 5). However, the long-term phase of persistent PKA activity, 20 hr after the end of massed 5-HT stimulation, was comparable to that observed after spaced application of 5-HT (compare Figures 3 and 5).

In summary, as shown in Figure 6, massed and spaced applications of 5-HT induce different patterns of persistent PKA activation: (1) spaced stimulation of the sensory neurons induces both an intermediate-term (1 hr) phase of persistent PKA activity that returns to baseline after 3 hr and a second long-term phase of persistent PKA activity at 20 hr, and (2) massed stimulation only induces the long-term phase of persistent PKA activity. Although both patterns of 5-HT application can induce long-term changes in PKA activity (Greenberg et al., 1987; Bergold et al., 1990; Hegde et al., 1997) as well as LTF in the sensory neurons (Emptage and Carew, 1993; Ghirardi et al., 1995; Zhang et al., 1997), our findings provide the first evidence that spaced and massed exposure of 5-HT can induce distinct phases of PKA activation.

Different Molecular Mechanisms Underlie the Distinct Phases of Persistent PKA Activity

The findings described above raise the interesting question of whether different molecular mechanisms contribute to the distinct phases of persistent activation of
blockers on 5-HT-induced transient PKA activation during the application of 5-HT.

As shown in Figure 7, long-term persistent PKA activity induced by spaced or massed application of 5-HT was completely abolished by inhibitors of protein or RNA synthesis, while at the same time, transient PKA activation induced by the test pulse of 5-HT and maximal PKA activity induced by BrAAMP (data not shown) were unaffected. In contrast, intermediate-term persistent PKA activation, which is induced only by spaced (but not by massed) exposure to 5-HT, was blocked by inhibitors of protein synthesis but not by blockers of RNA synthesis. Thus, the induction of the intermediate-term PKA activation depends on translation but not on transcription. These findings provide additional evidence that different mechanisms underlie intermediate-term and long-term persistent PKA activation induced by 5-HT.

Since intermediate-term persistent PKA activity depends on translation and is gradually induced after the fourth and fifth 5-HT pulses (Figure 2), we further analyzed the time window during which this form of activation is sensitive to inhibition of protein synthesis. As shown in Figure 8, addition of anisomycin during the first half of spaced 5-HT application leads to a total blockade of the intermediate-term persistent PKA activity, whereas blocking protein synthesis in the second half of spaced 5-HT application has no effect on the induction of persistent PKA activity. Thus, for the formation of the intermediate-term persistent PKA activity, translation was required only during the first few pulses of 5-HT. Such a temporally constrained requirement of translation was not found for the induction of long-term persistent PKA activity. Thus, translation during both the first and the second half of spaced 5-HT pulses was required for the induction of the persistent PKA activity after 20 hr. The same held for massed application of 5-HT for 90 min: translation was required throughout the duration of the 5-HT pulse to induce persistent PKA activation at 20 hr (Figure 8). These results, together with the fact that both repeated 5-HT pulses as well as prolonged, continuous 5-HT exposure lead to long-term

**Figure 6. Distinct Phases of Persistent PKA Activity Are Induced by Different Patterns of 5-HT Exposure**

Persistent PKA activation at different times is shown after either continuous (90 min) or 5 repeated pulses of 5-HT, with an intertrial interval of 15 min. Repeated pulses (black circles) induce intermediate-term (1 hr) and long-term (20 hr) persistent PKA activation, whereas continuous exposure (shaded circles) induces only the long-term phase. Note the biphasic profile of activation induced by repeated pulses: PKA activity is elevated at 1 hr, returns to baseline at 3 hr, and is once again elevated at 20 hr.

**Figure 7. Dependence of Persistent PKA Activation on Transcription and Translation**

Thirty minutes prior to 5-HT exposure, pleural-pedal ganglia were incubated in (1) ASW alone (contr), (2) anisomycin (aniso; 10 μM), or (3) actinomycin (actino; 50 μg/ml). The ganglia were exposed either to 5 pulses (5 min) of 5-HT or to a continuous application for 90 min. Thirty minutes after 5-HT exposure, the blockers were removed from the solution. Persistent PKA activity and transient PKA activity induced by a single pulse of 5-HT (5 min [Figure 3] or 90 min [Figure 5]) is not affected by anisomycin or by actinomycin (data not shown). Thus, we can exclude a direct effect of the
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Discussion

In the sensory neurons of Aplysia, 5-HT induces at least three temporally and mechanistically distinct phases of synaptic facilitation. A single pulse of 5-HT induces STF that lasts for about 15 min (Mauelshagen et al., 1996; Schacher et al., 1997). This form of facilitation is mediated by covalent modification of existing proteins (Sweatt and Kandel, 1989), primarily through the actions of two second messenger systems, PKA and PKC (reviewed by Byrne and Kandel, 1996). Repeated pulses of 5-HT induce a second phase, ITF, that lasts for 1-3 hr (Ghirardi et al., 1995; Mauelshagen et al., 1996; Hegde et al., 1997). This phase requires protein but not RNA synthesis (Ghirardi et al., 1995). Finally, the same repeated exposure to 5-HT that gives rise to ITF also induces a third phase, LTF, that lasts for 24 hr or longer (Montarolo et al., 1986; Clark and Kandel, 1993; Emptage and Carew, 1993; Zhang et al., 1997). This phase of facilitation requires synthesis of both mRNA and protein (Montarolo et al., 1986; Ghirardi et al., 1995; Martin et al., 1997).

In this paper, we show that 5-HT induces three comparable phases of PKA activation in the cell bodies of the sensory neurons. First, 1-3 pulses of 5-HT induce short-term PKA activation (ST-PKA) that lasts for about 5 min; this phase of activation requires neither RNA nor protein synthesis. Second, 5 pulses of 5-HT induce intermediate-term persistent activation of PKA (IT-PKA) that lasts for up to 3 hr; this phase requires protein but not RNA synthesis. Third, 5 pulses of 5-HT induce a long-term phase of persistent activation (LT-PKA) 20 hr later; this phase requires both RNA and protein synthesis.

The Three Phases of PKA Activation Are Independent

It has long been appreciated that 5-HT-induced short-term and long-term synaptic facilitation in the sensory neurons of Aplysia utilize different mechanisms. For example, Montarolo et al. (1986) first showed that LTF, but not STF, requires protein synthesis. Moreover, ITF can also be mechanistically distinguished from both STF and LTF. In cultured sensory neurons, Ghirardi et al. (1995) showed that STF, ITF, and LTF can be differentiated by the concentration of 5-HT required for their induction and by their underlying mechanisms: STF requires covalent modification, ITF requires translation, and LTF requires both transcription and translation. Previous work examining 5-HT-induced synaptic facilitation at the tail sensory to motor synapse in the intact CNS has also shown that STF and LTF are functionally independent. For example, the 5-HT antagonist yohimbine blocks the induction of STF (Mercer et al., 1991; Emptage and Carew, 1993) but does not interfere with the induction of LTF (Emptage and Carew, 1993). In addition, 5-HT applied exclusively to the soma of the sensory neurons induces LTF but not STF (Emptage and...
Spaced Stimulation

\[
\begin{align*}
\text{5-HT} & \quad \Rightarrow \quad \text{INTERMEDIATE-TERM} \\
\text{5-HT} & \quad \Rightarrow \quad \text{LONG-TERM}
\end{align*}
\]

Massed Stimulation

\[
\begin{align*}
\text{5-HT} & \quad \Rightarrow \quad \text{INTERMEDIATE-TERM} \\
\text{5-HT} & \quad \Rightarrow \quad \text{LONG-TERM}
\end{align*}
\]

Figure 9. Schematic Representation of Translational and Transcriptional Requirements for the Induction of Intermediate-Term and Long-Term Persistent PKA Activity in the Sensory Neurons

Intermediate-term persistent PKA activity induced by spaced 5-HT exposure requires translation, but only during the first 5-HT pulses. Massed 5-HT exposure does not induce intermediate-term PKA activation. Long-term persistent PKA activation, induced by either spaced or massed 5-HT application, requires translation and transcription throughout exposure to 5-HT.

Carew, 1993; for a related finding in siphon sensory neurons in the abdominal ganglion, see Clark and Kandel, 1993). Finally, as discussed above, STF and ITF decay to baseline hours before the initial expression of LTF. Thus, STF, ITF, and LTF in the tail sensory neurons can be functionally dissociated by pharmacological, anatomical, and kinetic criteria.

Just as 5-HT-induced synaptic facilitation can exist in several mechanistically distinct phases, likewise 5-HT-induced activation of the cAMP signaling cascade in the sensory neurons exhibits distinct phases. Brief 5-HT exposures induce transient elevation of cAMP activation in the sensory neurons (Bernier et al., 1982; Kandel and Schwartz, 1982), whereas prolonged exposures give rise to persistent cAMP activity that requires protein synthesis (Bergold et al., 1990). Our results support and extend these previous findings. Specifically, we have obtained three lines of evidence that are consistent with the hypothesis that ST-PKA, IT-PKA, and LT-PKA are mechanistically distinct. First, their induction requirements are different: ST-PKA requires neither translation nor transcription, IT-PKA requires translation but not transcription, and LT-PKA requires both translation and transcription. Second, as shown in Figure 9, the translational requirements for IT-PKA and LT-PKA differ: IT-PKA requires translation only during the early phase of 5-HT exposure (the first few pulses), whereas LT-PKA requires translation to occur throughout the entire exposure. Finally, LT-PKA can be expressed in the complete absence of IT-PKA; continuous (massed) stimulation by 5-HT induces LT-PKA that is dependent on both transcription and translation, but this same pattern of 5-HT exposure produces no IT-PKA (Figure 9).

On a mechanistic level, the long-term (20 hr) increase in persistent PKA activity is likely due to 5-HT-induced downregulation of the regulatory subunit of PKA that has been described by Schwartz and colleagues (Greenberg et al., 1987; Bergold et al., 1990; Chain et al., 1995; Hegde et al., 1997). Like the long-term change in PKA activity we observe, long-term downregulation of the regulatory subunit requires both transcription and translation (Bergold et al., 1990). If LT-PKA is dependent on the ubiquitin hydrolase pathway (Hegde et al., 1997), one might also predict that LT-PKA would be dependent on CREB activation (Bartsch et al., 1995).

While selective long-term proteolysis of the regulatory subunit provides a good candidate mechanism for the LT-PKA, the mechanisms underlying IT-PKA are less clear. Downregulation of the regulatory subunit of PKA as described by Schwartz and colleagues is not a likely mechanism, since it requires transcription as well as translation (Bergold et al., 1990), and we have shown that IT-PKA does not require transcription. The mechanisms underlying IT-PKA may well entail translationally sensitive changes in either activators or inhibitors of PKA itself or of other proteins that regulate PKA activity. An important feature of any mechanism underlying the induction of the IT-PKA will be its unique sensitivity to translation only during early stages of 5-HT exposure (Figures 8 and 9).

Taken collectively, the results examining 5-HT-induced plasticity in the sensory neurons reveal that several phases of facilitation can be induced at both synaptic and biochemical levels. As discussed above, many studies further show that several of these phases appear to be at least partly independent, utilizing different cellular and molecular mechanisms for their induction and expression. However, the apparent mechanistic independence of some of these processes, such as those underlying the different temporal phases of PKA activation in the present study, does not imply that these different phases cannot interact. In fact, as will be discussed below, a single brief exposure of 5-HT in the sensory neurons not only induces transient synaptic facilitation and PKA activation, but it can also set in motion a range of subcellular events that can interact with other cellular and molecular processes involved in the induction and maintenance of long-term synaptic modification.

Another issue raised by our results concerns the difference between continuous (massed) and spaced exposure to 5-HT. On a synaptic level, considerable evidence indicates that the pattern of 5-HT exposure can influence the induction of LTF. For example, the probability of inducing LTF is high following either spaced applications (Montarolo et al., 1986; Clark and Kandel, 1993; Emptage and Carew, 1993) or prolonged (>1 hr) continuous exposure (Emptage and Carew, 1993; Giriardi et al., 1995; Zhang et al., 1997) but is significantly reduced following briefer continuous exposure (e.g., 25 min; Mauelshagen et al., 1998). Our results are consistent with these observations: continuous, prolonged (90 min) exposure to 5-HT induces significant LT-PKA. The combined results described above elucidating the effects of 5-HT pattern both on the induction of LTF and LT-PKA now make it important to examine the degree to which behaviorally induced long-term sensitization in Aplysia is influenced by different patterns of training.
Phases of Activity in Aplysia Sensory Neurons

Trials, as is the case, for example, for long-term habituation in Aplysia (Carew et al., 1972) and for odor avoidance learning in Drosophila (Tully et al., 1994; see below).

Translational Events Must Interact with Other Signals in the Induction of Intermediate-Term PKA Activation

Our studies show that 5 pulses of 5-HT are required for the induction of IT-PKA. Translation of mRNA into protein is also required for IT-PKA induction, but it is only necessary during the first pulses of 5-HT (Figures 8 and 9). Blocking translation during later 5-HT pulses does not interfere with the induction of IT-PKA. These results thus show that translational events are initiated in the sensory neurons by early exposure to 5-HT, but these translational steps alone are insufficient to induce IT-PKA; they require additional combinatorial signals to lead to the induction of IT-PKA. What might these combinatorial signals be? In the present paper, we show that the additional signaling requirement can be met by further pulses of 5-HT. However, simple exposure to additional 5-HT is not enough, since continuous (massed) 5-HT exposure, giving rise to continuous PKA activation during training, does not induce IT-PKA. Rather, the additional 5-HT exposure must be comprised of spaced applications, indicating that some form of patterning (e.g., periods of PKA activation intercalated with periods of rest) is an important feature of this combinatorial signal in IT-PKA induction.

The above discussion indicates that spaced pulses of 5-HT can serve as a signal that interacts with early translational activation in the induction of IT-PKA. Recent evidence indicates that other signals might serve this function as well. For example, at the synaptic level, Sutton and colleagues (1998, Soc. Neurosci., abstract) have shown that direct activation of a sensory neuron during a single brief exposure to 5-HT gives rise to the induction of ITF. Since a comparable pulse of 5-HT alone does not induce ITF in the sensory neurons (Mauelshagen et al., 1996), these results show that some aspect of activity (e.g., Ca\(^{2+}\) influx; see, e.g., Hawkins et al., 1983; Walters and Byrne, 1983) might also serve as a combinatorial signal that can interact with 5-HT-induced translational events in the induction of ITF. Our suggestion that activity might serve as a combinatorial signal is not novel. There is considerable precedent that activity can serve as an important signal in the sensory neurons. For example, previous work has shown that sensory neuron activation coincident with 5-HT enhances the magnitude of STF (Hawkins et al., 1983; Walters and Byrne, 1983) and facilitates the induction of LTF (Schacher et al., 1997). Our suggestion serves to point to a specific kind of activity-dependent interaction, one that provides a necessary signal that combines with 5-HT-induced translational events in the induction of ITF. An important prediction from this suggestion is that activity in the sensory neurons should also enable 1-3 pulses of 5-HT to give rise to IT-PKA.

A more general consideration that arises from the discussion above is that brief modulatory signals which apparently induce only transient events, may in addition pave the way for further, more long-lasting plastic changes. However, the more lasting effects of a transient modulatory signal may often go undetected unless specific experimental attention reveals them. Several examples have arisen in the analysis of short-term and long-term plasticity in the sensory neurons of Aplysia that illustrate this point. First, Alberini and colleagues (1994) identified a transcription factor in the sensory neurons, ApC/EBP, that is rapidly induced by 5-HT and by cAMP, even in the presence of protein synthesis inhibitors. Blocking the function of ApC/EBP selectively blocked the induction of LTF in cultured sensory neurons without affecting STF. Alberini et al. (1994) found that within 15 min of 5-HT application there was a detectable increase in the level of ApC/EBP mRNA. Such a brief exposure to 5-HT is normally insufficient to induce LTF; nonetheless, this brief exposure is enough to set the synthesis of mRNA for ApC/EBP in motion. A second example is evident from the work of Bartsch and colleagues (1995), who cloned an inhibitory form of CREB in Aplysia (ApCREB2). Injection of an antibody against ApCREB2 into a cultured sensory neuron rendered a single brief pulse of 5-HT, which normally is insufficient to induce LTF (Montarolo et al., 1986; Emp-tage and Carew, 1993), capable of inducing both functional and structural long-term changes in the sensory neurons. Thus again, a brief exposure of 5-HT, in the appropriate molecular environment, is fully capable of inducing long-term plastic changes. A final example is evident from the work of Sherrf and Carew (1997, Soc. Neurosci., abstract) who took advantage of the observation that repeated spaced applications of 5-HT to tail sensory neurons in the intact CNS are significantly better than a single massed application in the induction of LTF (Mauelshagen et al., 1998). Sherrf and Carew (1997, Soc. Neurosci., abstract) found that massed (25 min) application of 5-HT exclusively to the sensory neuron soma did not induce LTF. However, if the somatic exposure was accompanied by a single brief pulse of 5-HT to the synapse (which itself does not give rise to LTF), LTF was induced. Thus, local, brief exposure of the synapse to 5-HT can interact with somatic events in the induction of LTF. Taken collectively, these results show that in the sensory neurons of Aplysia, a single brief exposure to 5-HT not only induces transient plasticity but also sets the stage for more lasting plasticity if additional combinatorial signals also occur.

Implications for Multiple Phases of Memory Storage

As discussed at the outset, numerous behavioral experiments have revealed that memory can exist in a wide range of temporal domains (McGaugh, 1966; Atkinson and Shiffrin, 1968; Byrne, 1983; Walters and Byrne, 1983) and facilitates the induction of LTF (Schacher et al., 1997). Our suggestion serves to point to a specific kind of activity-dependent interaction, one that provides a necessary signal that combines with 5-HT-induced translational events in the induction of ITF. An important prediction from this suggestion is that activity in the sensory neurons should also enable 1-3 pulses of 5-HT to give rise to IT-PKA.

A more general consideration that arises from the discussion above is that brief modulatory signals, which apparently induce only transient events, may in addition pave the way for further, more long-lasting plastic changes. However, the more lasting effects of a transient modulatory signal may often go undetected unless specific experimental attention reveals them. Several examples have arisen in the analysis of short-term and long-term plasticity in the sensory neurons of Aplysia that illustrate this point. First, Alberini and colleagues (1994) identified a transcription factor in the sensory neurons, ApC/EBP, that is rapidly induced by 5-HT and by cAMP, even in the presence of protein synthesis inhibitors. Blocking the function of ApC/EBP selectively blocked the induction of LTF in cultured sensory neurons without affecting STF. Alberini et al. (1994) found that within 15 min of 5-HT application there was a detectable increase in the level of ApC/EBP mRNA. Such a brief exposure to 5-HT is normally insufficient to induce LTF; nonetheless, this brief exposure is enough to set the synthesis of mRNA for ApC/EBP in motion. A second example is evident from the work of Bartsch and colleagues (1995), who cloned an inhibitory form of CREB in Aplysia (ApCREB2). Injection of an antibody against ApCREB2 into a cultured sensory neuron rendered a single brief pulse of 5-HT, which normally is insufficient to induce LTF (Montarolo et al., 1986; Emp-tage and Carew, 1993), capable of inducing both functional and structural long-term changes in the sensory neurons. Thus again, a brief exposure of 5-HT, in the appropriate molecular environment, is fully capable of inducing long-term plastic changes. A final example is evident from the work of Sherrf and Carew (1997, Soc. Neurosci., abstract) who took advantage of the observation that repeated spaced applications of 5-HT to tail sensory neurons in the intact CNS are significantly better than a single massed application in the induction of LTF (Mauelshagen et al., 1998). Sherrf and Carew (1997, Soc. Neurosci., abstract) found that massed (25 min) application of 5-HT exclusively to the sensory neuron soma did not induce LTF. However, if the somatic exposure was accompanied by a single brief pulse of 5-HT to the synapse (which itself does not give rise to LTF), LTF was induced. Thus, local, brief exposure of the synapse to 5-HT can interact with somatic events in the induction of LTF. Taken collectively, these results show that in the sensory neurons of Aplysia, a single brief exposure to 5-HT not only induces transient plasticity but also sets the stage for more lasting plasticity if additional combinatorial signals also occur.
to directly relate these cellular mechanisms to behavioral instances of different phases of memory processing in Aplysia. Recent studies in several other systems have made progress in this regard, by relating cellular and molecular plasticity to different phases of behavioral memory. For example, in an odor avoidance learning paradigm in Drosophila, Tully and colleagues (1994) have shown that spaced and massed training sessions give rise to two different forms of long-lasting memories that can be distinguished both genetically and by their dependence on protein synthesis. In addition, in classical conditioning of proboscis extension in the honeybee, which exhibits long-term retention following a variety of training procedures (see Hammer and Menzel, 1995), Müller (1996) has shown that memory at an identical retention interval can result from different mechanisms. Following multiple training trials, 24 hr retention requires the action of nitric oxide synthase, whereas context dependent retention following a single training conditioning trial does not. Finally, Abel and colleagues (1997) have shown that transgenic mice that express an inhibitory form of the regulatory subunit of PKA in hippocampus not only exhibit a reduction of hippocampal PKA activity and an impairment of the late phase of LTP in the CA1 region, but they also exhibit parallel behavioral deficits in spatial memory and long-term memory for contextual fear conditioning. These and related studies illustrate useful paradigms for relating different phases of molecular and biochemical activation to specific instances of behavioral memory.

In conclusion, studies in a number of systems have revealed important mechanisms underlying plasticity at behavioral, synaptic, genetic, cellular, and molecular levels of analysis. Considered together, these studies can provide valuable mechanistic criteria that may permit further identification of multiple, overlapping phases of memory that, collectively, account for the remarkable ability of all animals to encode experience.

Experimental Procedures

Preparation of the Ganglia

Wild-caught adult Aplysia californica (100-150 g) supplied from Marinus (Long Beach, CA) or Marine Specimens Unlimited (Pacific Palisades, CA) were used. Animals were anesthetized by injection of isotonic MgCl2 (100 ml/100 g body weight), and the pleural±pedal ganglia were dissected and desheathed in a Sylgard-coated dish per assay, of which at least 8 were assays from untreated controls. In each experiment, the mean specific PKA activity in the sensory neurons was measured by a fast in vitro phosphorylation assay using exogenously added I-1 as a PKA substrate (Hildebrandt and Müller, 1995a, 1995b). In initial experiments, we confirmed that I-1 purified from bovine brain is a specific substrate for cAMP-dependent protein kinase in Aplysia. For the phosphorylation assay, samples (10 µl) in the test tubes were thawed, and, just before complete melting, the phosphorylation mixture (30 µl) was added. The phosphorylation mixture contained 2 µCi [γ-32P]ATP (5000 Ci/mmol), 10 µM ATP, 5 mM EGTA, 10 mM mercaptoethanol in 50 mM Tris-HCl (pH 7.5), and an aliquot of the heat-stable I-1 (0.5 µg), boiled for 2 min prior to use. After incubation for 20 s at room temperature (20°C), reactions were stopped by adding 6 µl of sample buffer (0.25 M Tris-HCl [pH 6.8] containing 5% mercaptoethanol, 5% sodium dodecyl sulfate [SDS], 20% glycerol, and 0.1% bromphenol blue). SDS-PAGE and autoradiography were performed as described by Hildebrandt and Müller (1995a, 1995b). In each sample, the 32P incorporation into the externally added PKA-specific substrate I-1 was normalized with respect to the total 32P incorporation into intrinsic proteins (due to other kinase activities; see Figure 1A). Autoradiographs were scanned and the density of both the PKA-specific I-1 band and the bands of the intrinsic proteins was determined using NIH Image. To calibrate the film exposure, the 32P incorporation into the band of PKA-specific substrate I-1 and the other bands of intrinsic proteins was determined by a scintillation counter.

Data Analysis

To compensate for different numbers of sensory neurons in individual samples, the labeling of PKA-specific substrate I-1 was normalized with respect to the total labeling of intrinsic protein due to other kinase activities. This value represents the specific PKA activity of a specific sample. In order to assess both transient and persistent changes, the specific PKA activity in sensory neurons of stimulated ganglia was compared to that in the untreated contralateral ganglia in each experiment.

Each experiment consisted of 40 assays (80 sensory neurons per assay), of which at least 8 were assays from untreated controls (basal activity). For each experiment, the mean specific PKA activity of cells stimulated in situ with BcAMP was defined as maximal activity and assigned a value of 1.0. The mean of the values for the density of I-1 bands obtained from test groups was compared to the mean of the control groups. Data are reported as means ± SEM. Means were analyzed for normal distribution and equal variances, so that the significance of differences between the means could be tested by a Student's t test for independent means. All probability values are two tailed.

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