

Presynaptic Facilitation Revisited: State and Time Dependence

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The mechanisms underlying short-term presynaptic facilitation, the enhancement of transmitter release from sensory neurons in *Aplysia*, induced by serotonin (5-HT), can be divided into two categories: (1) changes in ionic conductances leading to spike broadening and enhancement of Ca^{2+} influx; and (2) actions on the machinery for transmitter release that are independent of spike broadening and the resulting increases in Ca^{2+} influx. Spike broadening and the associated enhancement of excitability are induced by the modulation of K^+ conductances in the sensory neuron. The cellular mechanisms that contribute to the enhancement of release that is independent of spike broadening are not known and may involve vesicle mobilization or other steps in exocytotic release. These two facilitatory actions of 5-HT are mediated by at least two second-messenger-activated protein kinase systems, protein kinase A (PKA) and protein kinase C (PKC).

These two second-messenger cascades overlap in their contributions to synaptic facilitation. However, their relative contributions to enhancement of transmitter release are not simply synergistic but are state- and time-dependent. The state dependence is a reflection of the synapse's previous history of activity. When the synapse is rested (and not depressed), a brief pulse of 5-HT (lasting from 10 sec to 5 min) produces its actions primarily through PKA via both spike broadening-

dependent and -independent mechanisms. The broadening primarily involves the modulation of a voltage-dependent K^+ current, I_{KV} , with a small contribution by a voltage-independent K^+ current, I_{KS} . By contrast, the enhancement of excitability is mediated primarily by the modulation of I_{KS} . As the synapse becomes depressed with repeated activity, the contribution of PKC becomes progressively more important. As is the case with PKA, PKC produces its action both by broadening the spike via modulation of I_{KV} and by a spike broadening-independent mechanism.

In addition to being state-dependent, the mechanisms of facilitation are time-dependent. There are differences in the response to 5-HT when it is given briefly to produce short-term facilitation or when the exposure is prolonged. When exposure is brief (≤ 5 min), PKA dominates. When exposure is prolonged (10–20 min), PKC becomes dominant as it is with depressed synapses. Thus, synaptic plasticity appears to be expressed in several overlapping time domains, and the transition between very short-term facilitation and various intermediate duration phases seems to involve interactive processes between the kinases.

Key words: synaptic facilitation; cAMP; 5-HT; K^+ channels; learning; *Aplysia*

The synaptic connections between the sensory neurons of *Aplysia* and their follower neurons in the gill- and tail-withdrawal reflexes undergo a variety of plastic changes in response to behavioral training procedures that give rise to associative and nonassociative forms of learning and to short- and long-term memory storage associated with each of these forms of learning. For that reason, these synaptic connections have been used as model systems for examining the elementary cellular mechanisms contributing to simple forms of learning and to short- and long-term memory storage (Kandel and Schwartz, 1982; Carew and Sahley, 1986; Byrne, 1987; Hawkins et al., 1993).

In this review, we focus on one form of plasticity in these synaptic connections, short-term heterosynaptic (presynaptic) facilitation, because this form of facilitation has been studied extensively and has provided several of the initial insights into

learning-related synaptic modulation (Carew and Sahley, 1986; Hawkins et al., 1986; Byrne, 1987). For example, studies of *Aplysia* synapses provided the first evidence for presynaptic (heterosynaptic) facilitation, the process by which activity in one (modulatory) pathway can facilitate transmitter release by another (test) pathway (Kandel and Tauc, 1965; Mintz and Korn, 1991; McGehee et al., 1995). This system also provided the first direct evidence that a second-messenger pathway [cAMP and the cAMP-dependent protein kinase A (PKA)] can regulate the strength of synaptic transmission (Brunelli et al., 1976), and that modulatory transmitters produce some of their actions on synaptic strength by phosphorylating ion channels (Siegelbaum et al., 1982; Shuster et al., 1985; Baxter and Byrne, 1990a). Finally, because the monosynaptic pathway in the gill- and siphon-withdrawal reflexes and the related pathway activated by tail stimuli belong to a reflex system that can be studied on the cellular level, the study of these systems in *Aplysia* has provided an exceptionally favorable opportunity for relating synaptic changes in specific cells to behavior and its development and to simple forms of learning (Byrne et al., 1993; Hawkins et al., 1993; Carew, 1995).

Studies carried out 30 years ago in the identified cell, R2, of the

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abdominal ganglion of *Aplysia* demonstrated that a strong synaptic input from one pathway produces heterosynaptic facilitation of transmission in another monosynaptic pathway that lasts for several minutes (Kandel and Tauc, 1965). Similar heterosynaptic facilitation was found later to be recruited for two forms of nonassociative learning in the gill-withdrawal reflex: dishabituation and sensitization (Castellucci et al., 1970; Carew et al., 1971). Sensitization is a form of learning in which an animal learns about the properties of a single, noxious stimulus. When a noxious stimulus is applied to the head or tail of *Aplysia*, the animal subsequently exhibits increased responsiveness in a variety of elicited defensive reflex responses. Dishabituation is a related form of learning in which the animal, after a noxious stimulus, exhibits enhanced responsiveness in a reflex that previously had been habituated and reduced in its responsiveness after a series of non-noxious stimuli.

Sensitization and dishabituation have been studied in some detail in three reflexes of *Aplysia*: (1) the gill-withdrawal reflex in response to siphon stimulation; (2) the tail-withdrawal reflex in response to tail stimulation; and (3) the siphon-withdrawal reflex in response to siphon stimulation. Both head and, more commonly, tail stimuli have been used as noxious stimuli to produce either sensitization or dishabituation. Thus, training for behavioral sensitization or dishabituation using tail (or head) shock leads to heterosynaptic facilitation of connections between identified sensory and motor neurons of the gill- and tail-withdrawal reflex (Castellucci et al., 1970; Kupfermann et al., 1970; Walters et al., 1983). Quantal analysis of evoked and spontaneous transmitter release at these synaptic connections, both in the ganglion and in dissociated cell culture, revealed that heterosynaptic facilitation is presynaptic in origin (Castellucci and Kandel, 1976; Dale et al., 1988) and can be induced by at least two different types of modulatory neurons that act on the sensory neurons: serotonergic neurons and the L29 cells, which release a transmitter that is still unidentified. The small cardiac peptide (SCP) also facilitates the transmission, but the cells that mediate this facilitation have not been identified. The serotonin (5-HT)-mediated transmission has been studied most extensively because it is particularly important. Reducing the intracellular levels of 5-HT by addition of the neurotoxin 5,7-dihydroxytryptamine (5–7 DHT) blocks the ability of tail stimuli to sensitize the gill-withdrawal reflex response (Glanzman et al., 1989). In addition, 5-HT simulates the actions of the tail stimulus, both in facilitating the strength of the connections between the sensory and motor neurons and in increasing strength of the reflex response (see Brunelli et al., 1976; Walters et al., 1983; Abrams et al., 1984).

Several studies have implicated cAMP as a second messenger in presynaptic facilitation. First, it was found that injection of cAMP or PKA into the presynaptic sensory neuron simulates the facilitation produced by tail shock or application of 5-HT (Brunelli et al., 1976; Castellucci et al., 1980; Klein, 1993). A similar simulation was produced by bath application of forskolin (Klein, 1993). Conversely, inhibitors of PKA block facilitation (Castellucci et al., 1982; Ghirardi et al., 1992). Second, it was found that tail stimuli or shock of peripheral nerves, as well as 5-HT (or SCP), produces an increase in cAMP levels in the sensory neurons (Bernier et al., 1982; Castellucci et al., 1982; Abrams et al., 1984; Ocorr and Byrne, 1985, 1986; Ocorr et al., 1985, 1986). Similar results on the intact ganglia were obtained earlier by Cedar and Schwartz (1972) and Cedar et al. (1972).

The facilitation produced by 5-HT and by tail stimuli was found to lead both to a broadening of the presynaptic spike and to an

enhancement in membrane excitability, as reflected by a lower threshold for the action potential and less frequency adaptation to a prolonged pulse of injected current (Klein and Kandel, 1978, 1980; Walters et al., 1983; Klein et al., 1986; Baxter and Byrne, 1990a). These findings prompted a search for the substrates of PKA that might contribute to this spike broadening and enhanced excitability. Using two-electrode voltage-clamp recordings, it was found that 5-HT and cAMP modulated a novel K^+ current that had only a slight voltage dependence (Klein et al., 1982; Pollock et al., 1985; Baxter and Byrne, 1989). Single-channel recordings identified a specific channel, the S (“serotonin”)-type K^+ channel, which is modulated by 5-HT, cAMP (Siegelbaum et al., 1982), and PKA (Shuster et al., 1985). The elementary conductance of the channel was not modulated by 5-HT; rather, 5-HT reduced the number of channels open in a given patch of membrane. Because 5-HT and tail stimuli produced a broadening of the spike and enhanced excitability, both of these changes were attributed to a reduction in the K^+ current through the S-type channel, designated I_{KS} (Klein et al., 1980, 1982).

These findings were combined to devise a cellular model for short-term facilitation (Klein and Kandel, 1980; Klein et al., 1980; Kandel and Schwartz, 1982; Baxter and Byrne, 1989) (see Fig. 1). According to this model, a single, brief sensitizing stimulus leads to the activation of facilitatory interneurons, resulting in the release of 5-HT (and other modulatory neurotransmitters), which produces presynaptic facilitation of the connections between sensory and motor neurons. 5-HT increases the rate of synthesis of cAMP, leading to the activation of PKA, phosphorylation of the S-type K^+ channels, and reduction of their conductance, g_{KS} . The reduction of I_{KS} via PKA leads to an increase in excitability of the sensory neurons, which is reflected in a greater number of spikes that is elicited in response to a 0.5–1 sec stimulus (data not shown in Fig. 1A) (Klein et al., 1986; Baxter and Byrne, 1990a; Ghirardi et al., 1992; Goldsmith and Abrams, 1992). In addition, each spike elicited in a sensory neuron after a sensitizing stimulus is broader (Fig. 1A3) and results in increased influx of Ca^{2+} via Ca^{2+} channels (g_{Ca}) and enhanced transmitter release. The enhanced transmitter release leads, in turn, to enhanced EPSPs in the motor neuron that could contribute (together with changes in other parts of the neural circuit) to an enhanced behavioral response (i.e., to sensitization) (Kandel and Schwartz, 1982).

This model was developed initially for presynaptic facilitation related to short-term sensitization. It was extended later to long-term sensitization (Kandel and Schwartz, 1982; Goebel et al., 1986; Zhang et al., 1994). Thus, whereas a single tail shock leads to reflex sensitization lasting minutes, four to five successive tail stimuli lead to sensitization lasting for more than a day; further stimulus repetition leads to sensitization lasting weeks. Both the functional facilitation of the sensory-to-motor synapse and its structural alterations—the growth of new synaptic connections—are correlated with long-term sensitization (Frost et al., 1985; Bailey and Chen, 1988a,b; Lee et al., 1995). Moreover, as is the case for short-term sensitization, long-term sensitization training can be simulated by applications of 5-HT (Montarolo et al., 1986; Emptage and Carew, 1993) or by stimulation of peripheral nerves (Zhang et al., 1994). Whereas a single, brief pulse of 5-HT produces short-term facilitation lasting minutes, five pulses of 5-HT produce long-term facilitation lasting 1 or 2 d. Long-term differs from short-term facilitation in requiring protein and RNA synthesis (Montarolo et al., 1986) and in giving rise to the growth of new synaptic connections (Bailey and Chen, 1988a,b; Glanzman et al., 1990). Nevertheless, long-term facilitation resembles

short-term facilitation in being induced by both 5-HT and cAMP and in involving both enhancement of transmitter release and enhanced presynaptic excitability (Dale et al., 1987). Moreover, the major current modulated during long-term facilitation appears to be I_{KS} (Scholz and Byrne, 1987). Thus, long-term facilitation seems to be an extension, in a protein synthesis- and RNA synthesis-dependent manner, of at least some aspects of short-term facilitation (Sweatt and Kandel, 1989). Independent support for the mechanistic relationship between short- and long-term facilitation has been obtained by the finding that both short- and long-term facilitation emerge at the same time during development, a time at which 5-HT already can activate the cAMP–PKA pathway fully but as yet cannot activate the protein kinase C (PKC) pathway (Carew et al., 1989).

Short-term facilitation, however, has proven to be more complex than long-term facilitation. Specifically, three additional components appear to contribute to short-term facilitation as a function of both the state of the synapses and the time after exposure to 5-HT. First, 5-HT has been found to modulate transmitter release independently of spike broadening (Gingrich and Byrne, 1984, 1985, 1987; Hochner et al., 1986b; Braha et al., 1990; Dale and Kandel, 1990; Ghirardi et al., 1992; Pieroni and Byrne, 1992; Sugita et al., 1992, 1994c; Klein, 1993, 1994a; Stark and Carew, 1994).

Second, short-term facilitation has been found to recruit an additional second-messenger system, PKC, as a function of synaptic state and time of exposure to 5-HT (Braha et al., 1990; Sacktor and Schwartz, 1990; Ghirardi et al., 1992; Sossin and Schwartz, 1992; Sugita et al., 1992, 1994a).

Third, aside from I_{KS} , other currents are modulated, one of which, the voltage-dependent current, I_{KV} , has been found to contribute critically to spike broadening (Baxter and Byrne, 1989, 1990a; Walsh and Byrne, 1989; Critz et al., 1991; Goldsmith and Abrams, 1992; Hochner and Kandel, 1992; Sugita et al., 1992, 1994a; Braha et al., 1993; White et al., 1994).

Here we review the experiments that have led to the findings of three additional steps in presynaptic facilitation and present an updated view (see Figs. 2, 3) of how 5-HT induces presynaptic facilitation as a function of the state of the synapse and of the time of exposure to 5-HT. On the basis of these findings, we then examine the new insights that have emerged as a result of experimental progress over the last decade.

DUAL CONTROL OF SYNAPTIC FACILITATION THROUGH SPIKE BROADENING-DEPENDENT AND -INDEPENDENT MECHANISMS

The initial clue to the existence of additional facilitatory mechanisms aside from spike broadening in short-term facilitation came from the discovery that the efficacy of mechanisms that contribute to presynaptic facilitation vary as functions of the state of the synapse. Although several critical aspects of the rested synapse could be explained qualitatively in the context of the model shown in Figure 1 (compare Figs. 1, 2A), additional mechanisms had to be postulated to explain facilitation of the synapse after repeated low-frequency homosynaptic activity in the presynaptic neuron.

The first evidence for this additional complexity came from mathematical modeling and computer simulations showing that once an action potential becomes greatly prolonged, further facilitation cannot occur by further spike broadening but must recruit a process in addition to spike broadening. These modeling studies suggested that spike broadening alone was insufficient to account fully for presynaptic facilitation and that this was partic-

An Early View of Presynaptic Facilitation

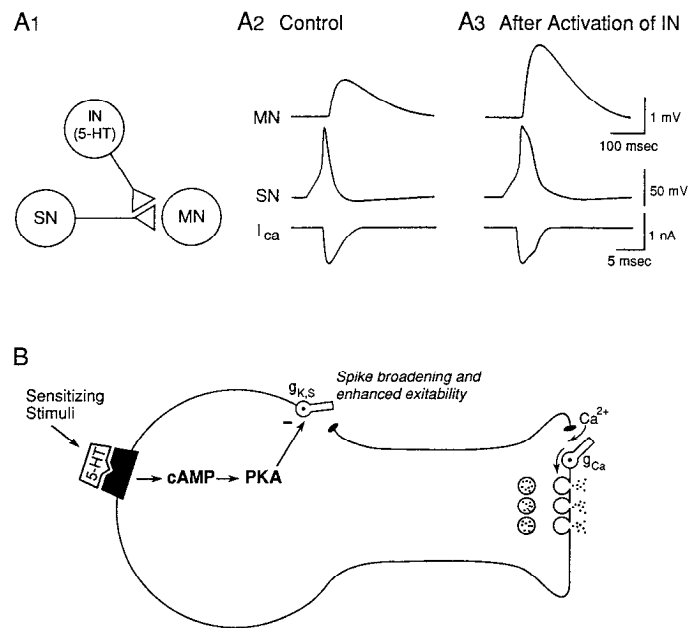


Figure 1. *A*, An early view of heterosynaptic facilitation that contributes to sensitization. *A1*, The synaptic connection between the sensory neuron (SN) and the motor neuron (MN) is enhanced by a facilitatory interneuron (IN) that releases 5-HT. *A2*, *A3*, Stylized representations of control and facilitated responses. *A2*, Control EPSP. An action potential in the SN leads to an influx of Ca^{2+} (I_{Ca}), the release of transmitter, and a subsequent EPSP in the MN. *A3*, EPSP produced by an action potential in the SN after neuron IN had been activated. The EPSP amplitude is larger because the facilitatory IN leads to a reduction in K^{+} conductance in the SN and a subsequent broadening of the action potential and enhancement of I_{Ca} caused by an increase in Ca^{2+} channel conductivity, g_{Ca} . The enhanced influx of Ca^{2+} leads to enhanced transmitter release and an enhanced EPSP. *B*, An early view of the molecular mechanisms underlying 5-HT-induced, short-term synaptic facilitation. In this scheme, cAMP is the only second messenger activated by 5-HT in the sensory neuron. cAMP-induced reduction of S-type K^{+} channel conductivity, $g_{K,S}$, is considered to underlie the modulation of two physiological properties of the sensory neuron: spike broadening and enhanced excitability. Spike broadening is believed to play a key role in synaptic facilitation of sensorimotor connections as shown in *A*.

ularly apparent when the demand on transmitter release becomes great. To maintain or augment the transmitter pool, 5-HT must act directly to mobilize transmitter vesicles to the active zone (Gingrich and Byrne, 1984, 1985, 1987; Gingrich et al., 1988).

The first experimental evidence for a second process in facilitation (one independent of spike broadening) came from experiments that demonstrated that 5-HT can produce facilitation independent of spike broadening when the demand on transmitter release becomes great (Hochner et al., 1986a,b). Explorations of how the duration of the presynaptic action potential, controlled by voltage-clamping the presynaptic cell, controls transmitter release in the presence or absence of 5-HT showed that in the absence of 5-HT, even increases of 10–30% in the duration of the action potential produced significant increases in EPSP amplitude, which is indicative of presynaptic facilitation. Preventing broadening of the presynaptic action potential by voltage-clamping the presynaptic neuron reduced the facilitation produced by 5-HT (Hochner et al., 1986a,b). These results were consistent with findings to emerge later in studies of the squid giant synapse that even slight

broadening in the presynaptic spike by 10 or 20% produces large increases (50–100%) in transmitter release (Augustine, 1990).

In the course of this work, it was discovered that an additional set of processes also contributes to facilitation (Hochner et al., 1986a,b). When the sensory neuron is activated repeatedly, it undergoes a striking decrease in synaptic strength, called “homosynaptic depression,” caused by a progressive decrease in the amount of transmitter released by each action potential. In the presence of homosynaptic depression, prolonging the duration of the depolarizing voltage-clamp command had little effect on transmitter release. Nevertheless, 5-HT was still capable of enhancing release. Moreover, once 5-HT enhanced release, increases in the duration of the presynaptic command again became highly effective in enhancing release further. Consistent with the mathematical model (Gingrich and Byrne, 1985, 1987), these studies suggested that when transmitter release becomes depressed (presumably because of a limited supply of transmitter) (Bailey and Chen, 1988c), 5-HT is capable of modulating some aspects of release not available to spike broadening alone. For spike broadening to increase release in depressed synapses, 5-HT first must recruit other processes necessary for facilitation. These findings suggested that two major processes contribute to facilitation at all times: (1) a spike-broadening process that leads to increased Ca^{2+} influx and, consequently, enhanced release; and (2) a Ca^{2+} -independent process related to some step in the release process, perhaps mobilization or exocytosis itself, that does not involve Ca^{2+} influx (Hochner et al., 1986a,b; Braha et al., 1990). Although the two processes were thought to have overlapping domains, spike broadening was thought to predominate in the nondepressed synapse, whereas the spike broadening-independent process was thought to become important as an additional step in facilitation at the depressed synapse.

Studies of learning traditionally had distinguished between *sensitization*, the enhancement of a reflex response by a noxious stimulus, and *dishabituation*, the enhancement by a noxious stimulus of a reflex that previously had been habituated. That a second process, independent of spike broadening and increased Ca^{2+} influx, contributes to presynaptic facilitation at the depressed synapse suggested that this behavioral distinction is also present at the cellular level (Hochner et al., 1986a). That parallel is now even more clearly visible by the finding that dishabituation and sensitization emerge as separate processes during development in *Aplysia* (Rankin and Carew, 1988). For example, short-term dishabituation of the gill-withdrawal reflex emerges at an earlier time in development than does short-term sensitization. Moreover, lower concentrations of 5-HT are required to produce facilitation of depressed synapses than are required to facilitate nondepressed synapses (Emptage and Carew, 1994; Emptage et al., in press). The mechanism for this differential effect of 5-HT is not known.

The finding that 5-HT activates mechanisms in addition to spike broadening for enhancing transmitter release led to a re-examination of the relationship between spike broadening and transmitter release. The initial correlation between transmitter release at the terminal and spike broadening was based on the recording of action potential duration (and later of Ca^{2+} influx) into the cell body of the presynaptic neuron (Blumenfeld et al., 1990). To obtain data directly from the presynaptic terminals rather than from the cell body, Eliot et al. (1993) studied synapses between sensory and motor neurons reconstructed in dissociated cell culture, imaging Ca^{2+} influx into the presynaptic terminals in

response to action potentials. Using synapses that received minimal previous stimulation, they found that 5-HT enhanced Ca^{2+} influx through dihydropyridine-insensitive Ca^{2+} channels near release sites in parallel with spike broadening. The influx was correlated with the degree of facilitation. Although the duration of the action potential in the presynaptic terminals could not be measured, parallel experiments in the cell body showed a direct relationship between the 5-HT-induced increase in Ca^{2+} influx and spike duration (Eliot et al., 1993). These results provided evidence that when spike broadening occurred, there was a sufficient increase in Ca^{2+} influx into the presynaptic terminals to contribute significantly to presynaptic facilitation.

Parallel studies of the relationship between somatic spike broadening and transmitter release were performed in intact pleural–pedal ganglia (Sugita et al., 1994c) and in dissociated cell culture (Klein, 1994a). Using low concentrations of K^{+} channel-blocking agents such as tetraethylammonium or 4-aminopyridine, (4-AP), these studies showed an approximately linear relation between the increase in spike duration and the increase in the amplitude of synaptic potentials. Moreover, there was a strong temporal correlation between the development of 4-AP-induced spike broadening and synaptic facilitation, which is consistent with the idea that spike broadening can contribute directly to facilitation of the synaptic connections.

The facilitation induced by 5-HT was found to develop rapidly and reach a peak within 3 min after its application. By contrast, the spike broadening induced by 5-HT did not reach its peak until 12 min after its application (Sugita et al., 1994c). Comparison of 4-AP-induced and 5-HT-induced spike duration and synaptic enhancement indicated that, even in the nondepressed synapse, a component of the observed 5-HT-induced facilitation at 3 and 6 min after application was independent of the increase in spike duration (Klein, 1994a; Sugita et al., 1994c).

These conclusions have been supported by studies indicating that depending on the precise experimental conditions, including the concentration of 5-HT and the duration of exposure, either of the two components for facilitation can dominate. Under some circumstances, facilitation of even nondepressed synapses can occur in the absence of spike broadening. For example, in the absence of action potentials, 5-HT leads to enhanced spontaneous transmitter release from sensory neurons in culture (Dale and Kandel, 1990). This enhanced release is independent of Ca^{2+} influx (see also Ghirardi et al., 1992). In a novel culture system in which sensory and motor neurons make direct soma-to-soma contacts, it was shown that 5-HT can produce cAMP-mediated facilitation that does not require spike broadening (Klein, 1993, 1994b). Similarly, during development 5-HT can induce facilitation and changes in excitability (presumably acting via cAMP), with only minimal spike broadening ($\leq 10\%$). Major spike broadening emerges later (Marcus and Carew, 1990, 1992; Stark and Carew, 1994).

These studies suggest that a spike broadening-independent process (perhaps a direct enhancement of transmitter mobilization or exocytosis) is a central mechanism for facilitation. Spike broadening seems to represent an additional amplification mechanism functioning to enhance the basic capability for facilitation. Although the two mechanisms can be dissociated with certain protocols, it is likely that both mechanisms contribute significantly to behavioral sensitization (Stark et al., in press).

RECRUITMENT BY 5-HT OF TWO SECOND MESSENGERS, PKA AND PKC, IN A STATE- AND TIME-DEPENDENT MANNER

The recruitment by 5-HT of a second facilitatory process, independent of spike broadening, led to the search for the second messengers that might mediate this process. PKC became a candidate for a second messenger when it was found that phorbol esters enhance release at the sensory neuron–motor neuron synapse and that they can do so even when release is very depressed (Hochner et al., 1985; Braha et al., 1990). In support of these findings, it was found that both sensitizing stimuli and 5-HT lead to the translocation to the membrane of the Ca^{2+} -dependent isoform of PKC, one of two isoforms of PKC present in sensory neurons (Sacktor and Schwartz, 1990; Kruger et al., 1991; Sossin and Schwartz, 1992; Braha et al., 1993; Sossin et al., 1993), both of which are activated by phorbol esters. cAMP did not translocate PKC, suggesting that the cAMP/PKA pathway and the PKC pathway are independent (Sacktor and Schwartz, 1990). These findings suggest that, in addition to PKA, presynaptic facilitation also involves PKC.

In dissociated cell culture, low concentrations of phorbol esters facilitate evoked release from the sensory neurons without causing spike broadening (Hochner et al., 1985; Braha et al., 1990), suggesting that at least one of the actions of PKC is a spike broadening-independent step in release. That the 5-HT-induced increase in spontaneous release (which occurs in culture in the absence of Ca^{2+} influx) is blocked by H7, a kinase inhibitor that appears to be relatively specific for PKC in *Aplysia* (Conn et al., 1989; Ghirardi et al., 1992), is consistent with this idea. By contrast, inhibitors of cAMP do not block the 5-HT-induced increase in spontaneous release (Ghirardi et al., 1992).

To test the relative contribution of the two kinases to nondepressed synapses and depressed synapses, the effects of inhibitors were examined. It was found that the degree to which the two kinases contribute to facilitation depends on the degree of the preexisting depression. When the synapse was not depressed, a single, 5 min pulse of 5-HT that produced short-term facilitation lasting minutes was blocked completely by inhibitors of PKA but was not affected by H7, an inhibitor of PKC. By contrast, as the synapse became more depressed, the inhibitor of PKC became progressively more effective in blocking 5-HT-induced short-term facilitation (Braha et al., 1990; Goldsmith and Abrams, 1991; Ghirardi et al., 1992). Finally, when the synapse is very depressed (to ~10% of control), the inhibitors of PKA have their least significant effect, blocking only 30% of the facilitation, whereas inhibitors of PKC block the facilitation almost completely. Independent evidence for the role of cAMP independent of spike broadening is provided by the facilitation by photorelease of “caged” cAMP of synapses that are moderately depressed to levels at which spike broadening is ineffective in enhancing release. In addition to these state-dependent effects, the ability of the inhibitors to attenuate 5-HT-induced facilitation was also dependent on the time after the application of 5-HT at which the synapse was tested. PKA inhibitors were more effective in attenuating 5-HT-induced facilitation on the first few responses after 5-HT application than were inhibitors of PKC (Braha et al., 1990; Hochner and Kandel, 1992). Conversely, Sugita et al. (1992) found that inhibitors of PKC were more effective in attenuating 5-HT-induced facilitation at later times.

Examination of the kinetics of spike broadening in response to a prolonged application of 5-HT showed that this process appears to have at least two components: a rapidly developing component

evident within 3–6 min, and a slowly developing component over the next 6–10 min (Hochner and Kandel, 1992; Stark et al., 1992; Sugita et al., 1992, 1994c; Stark et al., in press). Thus, a single, brief pulse of 5-HT lasting <5 min, typically used in cellular experiments to produce short-term facilitation, induces maximal facilitation but not maximal spike broadening. By contrast, at the end of a 12 min exposure to 5-HT, when the 5-HT-induced spike broadening had reached its peak, broadening was estimated to account for a greater percentage of the observed facilitation. These results suggested that although both spike broadening-dependent and -independent mechanisms can play important roles in the 5-HT-induced synaptic facilitation, their relative contributions vary not only with synaptic state but also with the total duration of exposure to 5-HT. These time-dependent effects are attributable to the differences in the kinetics of recruitment of PKA and PKC.

Thus, in the continuous presence of 5-HT over many minutes, inhibitors of PKA and PKC vary in their effectiveness in blocking spike broadening as a function of time after the continuous application of 5-HT. Consistent with the finding in cell culture that a 5 min pulse of 5-HT produces facilitation that is blocked by inhibitors of PKA, pre-exposure to staurosporine, an inhibitor of PKC, has little effect on 5-HT-induced spike broadening in the intact ganglion after a 3 min exposure of 5-HT. By contrast, staurosporine strongly inhibits the 5-HT-induced spike broadening at later times (Sugita et al., 1992).

MODULATION BY 5-HT OF TWO K^+ CURRENTS THAT PRODUCE SPIKE BROADENING AND INCREASED EXCITABILITY

The contribution of specific ionic currents to spike broadening was studied initially by simulating action potentials in the sensory neurons by applying depolarizing commands every 10–30 sec (Klein and Kandel, 1980; Klein et al., 1982; Pollock et al., 1985). With this protocol, at least three K^+ currents are activated: the S-type K^+ current (I_{KS}), the fast, transient outward current (I_{KA}), and the slower, transient-activated current (I_{KV}). However, I_{KV} exhibits cumulative inactivation and is mostly inactivated under this protocol. By stimulating less frequently, less inactivation of outward current is produced. Under these conditions, it was found that, in addition to modulating I_{KS} , 5-HT modulates I_{KV} , a current that is more important for spike broadening than I_{KS} (Baxter and Byrne, 1989). The modulation produced by 5-HT slows both the activation and the inactivation kinetics of I_{KV} (White et al., 1994). This causes a decrease in the net outward current elicited at the beginning of a voltage-clamp pulse and an increase at the end of the pulse. This effect develops slowly and becomes stable ~10 min after application of 5-HT.

Because of overlapping responses to electrical and pharmacological stimulation, it has been difficult to be certain of exactly how much the modulation of I_{KS} and I_{KV} contributes to spike broadening and to the excitability change. One evaluation of the relative contribution of I_{KS} and I_{KV} has come from an analysis of Hodgkin–Huxley-type mathematical models of the sensory neurons. These models indicated that I_{KS} plays a dominant role in regulating excitability but only a modest role in spike broadening. By contrast, I_{KV} plays a dominant role in regulating spike broadening (Baxter and Byrne, 1990b; Byrne et al., 1990; Canavier et al., 1991; Belkin et al., 1992).

Studies using activators and inhibitors of PKA and PKC indicated that the 5-HT-induced enhancement of excitability in sensory neurons is mediated almost exclusively by PKA (Klein et al.,

1986; Baxter and Byrne, 1990a; Ghirardi et al., 1992; Goldsmith and Abrams, 1992). 4β -Phorbol-12,13-diacetate, an activator of PKC, produces little or no enhancement of excitability (Braha, 1990; Sugita et al., 1992; Braha et al., 1993), and inhibitors of PKC do not inhibit the enhancement of excitability produced by 5-HT (Goldsmith and Abrams, 1992; Hohnner and Kandel, 1992; Sugita et al., 1992). By contrast, both PKA and PKC can produce a substantial spike broadening through I_{KV} equivalent to that produced by 5-HT. Conversely, the substantial spike broadening produced by 5-HT can be suppressed by inhibitors of either PKA or PKC (Goldsmith and Abrams, 1992; Sugita et al., 1992). Thus, whereas modification of excitability reflects the singular action of PKA, I_{KV} appears to be regulated jointly by PKA and PKC. However, the actions of the two kinases appear to vary as a function of the time of exposure to 5-HT (Braha et al., 1993). The modulatory action of cAMP on I_{KV} is fast, and a major part of the rapidly developing component of spike broadening appears to be dependent on PKA (Baxter and Byrne, 1990a; Ghirardi et al., 1992; Hohnner and Kandel, 1992). For example, a PKA inhibitor blocks 75% of this rapidly developing component, and a PKC inhibitor blocks 25% of it (Goldsmith and Abrams, 1992; Hohnner and Kandel, 1992; Sugita et al., 1992). In addition, intracellular injection of an analog of cAMP, Sp-cAMPS, or extracellular application of a membrane-permeable cAMP analog, 8-bromo-cAMP, produces spike broadening comparable with that induced by 5-HT within 3 min (Baxter and Byrne, 1990a; Hohnner and Kandel, 1992; Sugita et al., 1994b). By contrast, the modulatory effects of PKC on I_{KV} develop slowly and become stable more than ~10 min after its application (Sugita et al., 1994a).

Thus, whereas the rapid early action of 5-HT is mediated to a large degree by PKA, the slowly developing component of 5-HT-induced spike broadening evident after continuous exposure to 5-HT for >5 min is attributable primarily to the modulation of I_{KV} by PKC. This slow component of spike broadening is well mimicked by phorbol esters and is blocked by staurosporine (Marcus and Carew, 1992; Sugita et al., 1992).

We have focused only on two currents, I_{KV} and I_{KS} , because they have an important role in facilitation. But both PKA and PKC also modulate other currents in the sensory neuron. These include a slow component of I_{KCa} , designated I_{KCaS} , modulated by PKA (Walsh and Byrne, 1989; Goldsmith and Abrams, 1992; Hohnner and Kandel, 1992; Sugita et al., 1994a). Modulation of I_{KCaS} makes only a small contribution to 5-HT-induced enhancement of excitability (Canavier et al., 1991). Similarly, 5-HT and PKC cause increases in a nifedipine-sensitive Ca^{2+} current (Edmonds et al., 1990; Braha et al., 1993). Although modulation of this current is relatively small compared with the modulation of I_{KV} , it is plausible that an increase in Ca^{2+} current also contributes to spike broadening. The modulation of this current by 5-HT is slow to develop (Boyle et al., 1984; Braha et al., 1993; Klein, 1994b).

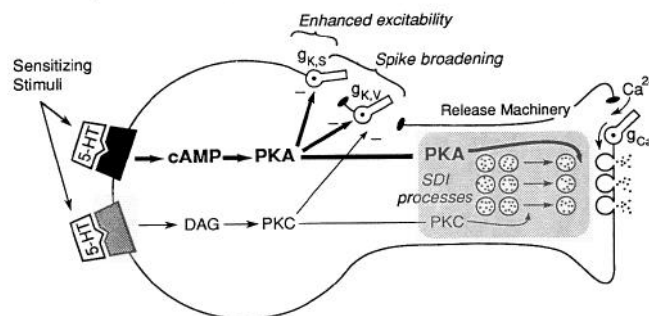
AN UPDATED MODEL FOR SHORT-TERM PRESYNAPTIC FACILITATION

In the process of testing and updating the models of the 1980s, several important new insights have emerged. These are summarized in Figures 2 and 3, which update our understanding of short-term facilitation.

At least two second-messenger protein kinases contribute to enhancement of transmitter release: PKA and PKC (Braha et al., 1990, 1993; Sugita et al., 1992). The existence of these two second-messenger-activated kinases implies that there are two different

State Dependence

A Non-Depressed Synapse



B Depressed Synapse

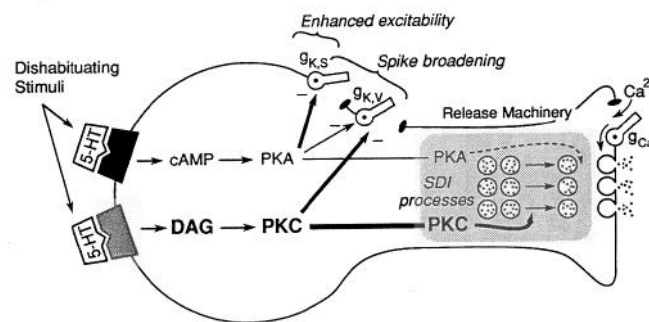


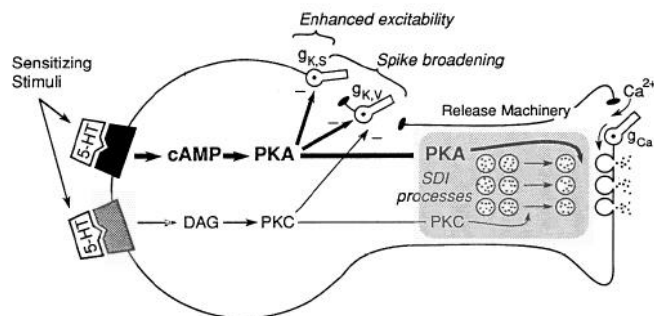
Figure 2. Current model of a sensory neuron that depicts the multiple processes for facilitation and their state dependence. 5-HT released from the facilitatory neuron (Fig. 1A1) binds to at least two distinct classes of receptors on the outer surface of the membrane and leads to the transient activation of two intracellular second messengers, DAG and cAMP. These second messengers, acting through their respective kinases (PKC and PKA), affect multiple cellular processes, the combined effects of which lead to enhanced transmitter release when a subsequent action potential is fired in the sensory neuron. *A*, In nondepressed synapses, the cAMP-signaling pathway plays a predominant role in facilitation through modulation of membrane currents and through modulation of a spike-duration independent (SDI) process that presumably involves some aspect of the release mechanism itself. *B*, In synapses depressed by previous activity, the DAG-signaling pathway plays a dominant role in facilitation by engaging the SDI process and mobilizing vesicles from a reserve or storage pool. PKA cannot facilitate a depressed synapse. Thus, PKC and PKA appear to affect the SDI process at different loci. Positive (+) and negative (−) signs indicate enhancement and suppression of cellular processes, respectively. Thin lines and smaller lettering indicate weaker effects than solid lines and larger lettering.

second-messenger-synthesizing enzymes and presumably different guanosine triphosphate-binding proteins (Fig. 2A). This is most consistent with the suggestion that 5-HT acts on at least two types of 5-HT receptors of the sensory neuron (Mercer et al., 1991). One receptor is thought to be coupled to adenylyl cyclase, leading to an increase in the level of cAMP and the activation of a PKA pathway (Bernier et al., 1982; Abrams et al., 1984; Ocorr and Byrne, 1985; Ocorr et al., 1986; Bacskaï et al., 1993). The other receptor presumably is coupled to phospholipase C, causing the formation of diacylglycerol (DAG) and the activation of PKC (Braha, 1990; Sacktor and Schwartz, 1990; Sugita et al., 1992; Braha et al., 1993).

These two kinases have overlapping spheres of action. Both act on transmitter release in two ways: (1) via a Ca^{2+} -dependent

Time Dependence

A Brief exposure to 5-HT



B Prolonged exposure to 5-HT

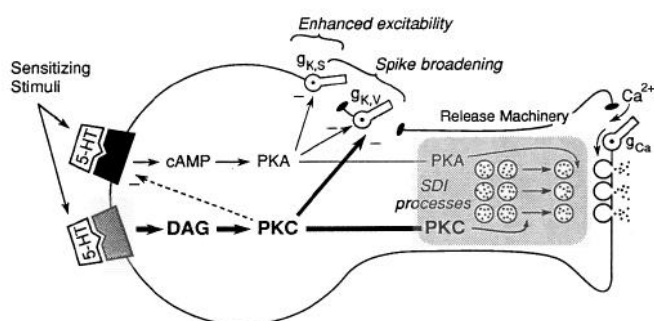


Figure 3. Current model for the time dependence of short-term synaptic facilitation produced by 5-HT. *A*, At early times and with brief applications (<5 min), PKA plays a primary role in 5-HT-induced synaptic facilitation through the modulation of membrane currents and the SDI processes. *B*, At later times and with more prolonged exposure to 5-HT, PKC plays a dominant role in 5-HT-induced maintenance of facilitation by spike broadening via the modulation of I_{KV} and the SDI processes. Recent results suggest that PKC can downregulate the 5-HT-induced increase in cAMP, perhaps via action on the receptor coupled to adenylyl cyclase (dashed line).

process resulting from spike broadening; and (2) via a spike duration-independent (SDI) mechanism.

We now have a good understanding of the mechanisms contributing to spike broadening and the related excitability change. Spike broadening is mediated by two major currents, predominantly by I_{KV} with a minor contribution from I_{KS} . In addition, I_{KS} mediates all (or almost all) of the excitability change. Whereas I_{KV} is modulated by both PKA and PKC, I_{KS} is modulated only by PKA.

By contrast, we still do not know how the spike broadening-independent facilitation mechanisms work. One possibility is that PKA and PKC have a direct action on vesicle mobilization or exocytosis. Alternatively, either or both of these two kinases could modulate as yet uncharacterized Ca^{2+} channels localized to the active zones (Klein, 1994b). Independent of its detailed mechanisms, this spike broadening-independent mechanism appears to be an important component of facilitation. The modulation of spike duration seems to serve as an additional step to amplify release further (Fig. 24).

The finding that the two protein kinases, which contribute to presynaptic facilitation, do not simply act interchangeably or

synergistically but contribute in a state- and time-dependent manner is of particular interest. This dependence is important not only from a cell biological point of view, but it is potentially important also from a behavioral perspective. It has been known for some time that even simple forms of learning, such as dishabituation and sensitization, are highly dependent on the parameters of the particular paradigm used, such as the intensity and duration of the test and sensitizing stimuli. The studies of presynaptic facilitation illustrate, on the level of a single synaptic connection, that cellular processes contributing to dishabituation and sensitization are themselves dependent on state and time.

The surprising discovery that facilitation is state-dependent has introduced a novel dimension into our thinking about synaptic plasticity, a dimension that is particularly interesting considering its parallel to the state dependence of the reflex enhancement that gives rise to sensitization or dishabituation. Thus, in response to a single, brief tail shock or a single, brief pulse of 5-HT lasting ≤ 5 min, the synaptic facilitation of a nondepressed synapse recruited by sensitization appears to be mediated primarily by the cAMP/PKA pathway (Ghirardi et al., 1992; Sugita et al., 1994c). Such facilitation is well simulated by activators of PKA (forskolin) and analogs of cAMP (Ghirardi et al., 1992; Klein, 1993); this facilitation is blocked by inhibitors of PKA, and it is unaffected by inhibitors of PKC (Ghirardi et al., 1992). This dependence on PKA is consistent with the finding that 5-HT produces a rapid increase in the concentration of cAMP in the sensory neurons, which reaches a peak within 2–3 min (Bacskai et al., 1993). The facilitation of the nondepressed synapses by PKA involves both a spike duration-independent and a spike duration-dependent process (Fig. 24) (Gingrich and Byrne, 1984, 1985; Sugita et al., 1992; Klein, 1993, 1994a).

By contrast, the facilitation of depressed synapses recruited for dishabituation relies heavily on PKC (Braha et al., 1990; Ghirardi et al., 1992). With moderate levels of depression, both kinases seem to be equally involved. On the other hand, facilitation of synapses depressed by 90% is largely attributable to PKC (Fig. 2B).

As with all aspects of behavior, it has been impossible in *Aplysia* to explore all possible parameters of sensitization, dishabituation, and 5-HT-induced facilitation. The standard protocol used for short-term sensitization has been a single train of stimulation to the body or to a peripheral nerve, typically 10 Hz for 10–15 sec. This stimulation produces short-term sensitization or presynaptic facilitation in the intact animal or in the isolated ganglion. Alternatively, a single pulse of 5-HT applied from 10 sec to 5 min is used often to produce short-term facilitation. Long-term sensitization requires four to five repetitions of the sensitizing train or of the pulse of 5-HT, and repetitions typically are spaced with 20 min intervening separations. Thus, most of the protocols that use 5-HT to examine facilitation have used pulses of 5 min or less. However, facilitation also is time-dependent (Sugita et al., 1994c). When 5-HT is present continuously for >5 min (10–30 min), as might be the case in some protocols that produce long-term facilitation, the balance between processes changes. This prolonged-exposure protocol has helped us to understand the dynamics of the coordination between PKA and PKC, a coordination that may be particularly important for intermediate durations of memory storage. This is particularly interesting considering the suggestion that PKC has a role in an intermediate phase that bridges short- and long-term facilitation (Sossin et al., 1994).

This time dependence of facilitation (Fig. 3) is supported by independent biochemical studies showing that different lengths of exposure to 5-HT lead to the phosphorylation of different sets of substrate proteins. Quantitative two-dimensional PAGE of ^{32}P -labeled sensory neurons showed that each of 18 specific proteins found to be significantly phosphorylated after 5 min exposure to 5-HT also was phosphorylated by application of cAMP, and only 2 of these proteins were phosphorylated also by application of the phorbol ester PDAc (Sweatt and Kandel, 1989). In a separate study, it was found similarly that after 2 min exposure to 5-HT the phosphorylation of one protein was affected by cAMP, whereas none was affected by PDAc (R. Homayouni, J. Byrne, and A. Eskin, unpublished observations). By contrast, after exposure to 5-HT ranging from 25 min to 1.5 hr, proteins also were affected by PDAc (Homayouni et al., 1995). Thus, whereas the PKA pathway appears to be involved in the early effects of 5-HT on protein phosphorylation, PKC seems to be involved at later times.

AN OVERVIEW

The first 15 years of studying presynaptic facilitation outlined its role in sensitization and dishabituation in the gill-withdrawal reflex, delineated its presynaptic nature, and documented the participation of cAMP and PKA in ion channel modulation and in the enhancement of transmitter release. The last 15 years of studies have elaborated on the detailed intracellular mechanisms involved. These recent studies have provided evidence for the coordinated participation of two different second-messenger pathways, PKC and PKA, in regulating the enhancement of transmitter release and have delineated the specific role of each of these second-messenger pathways in its contribution to facilitation. In addition, these recent studies have characterized the two major K^+ currents that contribute to spike broadening and have led to the discovery that, in addition to spike broadening, presynaptic facilitation involves a direct modulation of one or another step in transmitter mobilization or release.

However, despite the progress made during the last decade in analyzing the mechanisms of short-term presynaptic facilitation using certain specified protocols, the precise quantitative contributions of the various processes that participate in facilitation under a wider range of conditions continue to be difficult to assess. This is because the processes contributing to facilitation, much as those that contribute to hippocampal long-term potentiation (LTP), are highly dependent on the precise pattern of stimulation. In addition, the reagents used so far to isolate and study these processes have been less than ideal. For example, in *Aplysia* neurons 5-HT leads to the translocation of only one isoform of PKC, a Ca^{2+} -dependent isoform. By contrast, phorbol esters, which have been used to activate PKC, translocate both isoforms. Similarly, spike broadening and the spike broadening-independent modulation of release are complex processes that vary as functions of time and concentration of 5-HT. Also, the processes that take place in the synaptic terminal may differ from those that are measured in the soma. In addition, the relative recruitment of the component processes also may vary as a function of temperature and experimental preparation (intact ganglion or various types of dissociated cell culture). Each of these experimental systems, although significantly related to the other systems it is designed to model qualitatively, nevertheless is likely to differ from the other systems quantitatively in the extent of spatial

distribution of receptors and ion channels, complements of biochemical cascades, and surface-to-volume ratios.

It will be particularly informative, therefore, to explore directly the molecular cross-talk between the various second-messenger and protein kinase systems. For example, recent results suggest that PKC can reduce the 5-HT-induced increase in cAMP, perhaps by acting on the receptor coupled to adenylyl cyclase (Sugita et al., 1993). How does this occur? Does PKC activate a β -adrenergic receptor kinase-like kinase that phosphorylates and desensitizes the 5-HT receptor coupled to adenylyl cyclase? Recent results also indicate that, in addition to PKA and PKC, 5-HT recruits the Ca^{2+} /calmodulin-dependent protein kinase II (Saitoh and Schwartz, 1983; Nakanishi et al., 1994) and the microtubule-associated protein kinase (Michael et al., 1995). Finally, it will be important to relate the contribution of these monosynaptic pathways to gill, siphon, and tail withdrawal under a variety of learning conditions.

Despite these quantitative difficulties and the gaps in our knowledge, we believe that the updated models of Figures 2 and 3 accurately describe our current understanding of presynaptic facilitation in at least qualitative terms. As the initial cell biological models of the early 1980s served as a starting point for a generation of studies on the biophysical level relating the properties of single cells to behavior, we hope that these updated views will stimulate a new round of explorations, this time on a molecular level. Moreover, the finding that at the sensory-to-motor synapse facilitation is state- and time-dependent seems to be emerging as a general feature of synaptic plasticity. For example, synaptic plasticity in the CA1 region of the hippocampus involves multiple kinase and phosphatase systems, and the qualitative nature of the change (long-term depression, depotentiation, or LTP) is dependent on the state of the synapse. Similarly, in LTP there are multiple temporal domains of facilitation (Huang and Kandel, 1994; Huang et al., 1994). Thus, it will be important in the future to determine the molecular logic and behavioral relevance of these state- and time-dependent processes for various types of learning.

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