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# Tail shock produces inhibition as well as sensitization of the siphon-withdrawal reflex of *Aplysia*: Possible behavioral role for presynaptic inhibition mediated by the peptide Phe-Met-Arg-Phe-$NH_2$

**(behavioral inhibition/synaptic inhibition/identified neuron)**

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**ABSTRACT Recent studies have shown that, in addition to being modulated by presynaptic facilitation, the sensory neurons of the gill- and siphon-withdrawal reflex of *Aplysia* are also capable of being modulated by transient presynaptic inhibition produced by the peptide Phe-Met-Arg-Phe-$NH_2$. These two modulatory effects involve different second-messenger systems: the facilitation is mediated through cAMP-dependent protein phosphorylation, and the inhibition is mediated through the lipoxygenase pathway of arachidonic acid. To explore the behavioral function of this inhibition, we have carried out a parametric analysis of the effect of tail shock on the siphon-withdrawal reflex. In addition to producing sensitization of the withdrawal reflex, tail shock also transiently inhibits the reflex. The inhibition is produced by relatively weak shock, whereas sensitization is more prominent and may mask the inhibition with stronger shock. Furthermore, inhibition is not observed after habituation training. Cellular studies suggest that the behavioral inhibition is mediated, at least in part, by presynaptic inhibition of transmitter release from the siphon sensory neurons. Moreover, we have identified an interneuron within the left pleural ganglion (LPL16) that shows Phe-Met-Arg-Phe-$NH_2$ immunoreactivity, is activated by tail shock, and simulates the presynaptic inhibitory actions produced by tail shock. Therefore, our results suggest that presynaptic inhibition mediated by Phe-Met-Arg-Phe-$NH_2$ and its lipoxygenase second messenger contributes to behavioral inhibition of the siphon-withdrawal reflex.**

Modern studies of the properties of stimuli that serve as reinforcers for sensitization and classical conditioning in vertebrate learning reveal that these stimuli usually have two components, a prominent facilitatory component and a less-obvious inhibitory component. The facilitatory component is important for sensitization and conventional classical conditioning, whereas the inhibitory component is important for conditioned inhibition (1, 2). These dual properties of unconditioned stimuli have also been studied recently in invertebrates (6, 14, 20–26, 35). We describe here the existence of an inhibitory component of the unconditioned stimulus for learning in *Aplysia* and show that this component appears to use Phe-Met-Arg-Phe-$NH_2$ as one of its transmitters.

An aversive stimulus such as an electrical shock delivered to the tail of *Aplysia* leads to a learned behavioral enhancement or sensitization of the gill- and siphon-withdrawal reflex to siphon stimulation (3–5). If the tail stimulus is paired with a stimulus to the siphon, the reflex undergoes classical conditioning (6–8). Both the sensitization and classical conditioning are due in part to facilitation of transmitter release from siphon sensory neurons onto their follower neurons, including gill and siphon motor neurons (9–11). This presynaptic facilitation is mediated in turn by serotonergic and other facilitatory interneurons activated by tail shock (12–17). The facilitatory transmitters modulate release from the sensory neurons by broadening the action potential and by mobilizing available transmitter within the terminals of sensory neurons (18, 19).

In addition to these facilitatory actions, two earlier strands of work suggested the possibility that aversive stimuli in *Aplysia* also have inhibitory capabilities. First, earlier behavioral studies of sensitization and of classical conditioning showed that the sensitization produced by tail shock is often delayed in its maximal expression, as if the tail shock also initiates an inhibitory process that then decays more rapidly than the sensitization (6). More recently, Carew and his collaborators (20, 21) have obtained direct evidence that tail shock activates an early inhibitory process in juvenile and adult *Aplysia*. Second, cellular physiological studies have demonstrated that dopamine and the peptides Phe-Met-Arg-Phe-$NH_2$ and arginine vasotocin produce presynaptic inhibition of the siphon sensory neurons (14, 22, 23). The inhibitory action of Phe-Met-Arg-Phe-$NH_2$ has been studied in detail and has been found to involve hyperpolarization of the sensory neurons and narrowing of action potentials, caused by an increase in the probability of opening specific $K^+$ channels designated S and probably also a decrease in the $Ca^{2+}$ current (24, 25). These actions are mediated by a novel second messenger system, the lipoxygenase metabolites of arachidonic acid (26).

We have now investigated inhibition of the siphon withdrawal reflex on the behavioral and cellular levels and provide evidence here that Phe-Met-Arg-Phe-$NH_2$, presumably acting through the arachidonic acid cascade, plays a role in mediating behavioral inhibition.

## MATERIALS AND METHODS

Standard behavioral (7, 8) and electrophysiological (11, 12) procedures were used. The immunocytochemical procedure was slightly modified from the whole-mount technique of Longley and Longley (27). Rabbit anti-Phe-Met-Arg-Phe-$NH_2$ antisera was obtained from Immuno Nuclear (Stillwater, MN) and rhodamine-labeled goat anti-rabbit antiserum was obtained from Cappel Laboratories (Cochranville, PA). The tissue was viewed with filter packs D and N on a Leitz fluorescence microscope.

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Abbreviations: $Et_4N^+$, tetraethylammonium chloride; EPSP, excitatory postsynaptic potential; US, unconditioned stimulus; CS, conditioned stimulus; ITI, intertrial interval; df, degrees of freedom.

## RESULTS

**Aversive Stimulation Produces Inhibition as Well as Sensitization of the Siphon-Withdrawal Reflex.** Tail shock produces short- and long-term sensitization of the siphon-withdrawal reflex (3–5). In the experiments reported here, we investigated the conditions under which the reflex also undergoes behavioral inhibition by varying shock intensity, the time between shock and testing, and the number of pretests. In so doing we modified our usual protocol so as to detect transient changes in reflex responsiveness under conditions of minimal habituation, as suggested by Carew and colleagues (20, 21).

We first investigated the effect of shock intensity, using three groups of animals (Fig. 1A). One group received weak shock (10 mA), a second group received strong shock (100 mA), and a control group received no shock. We measured the duration of siphon withdrawal in response to weak tactile stimulation of the siphon once every 15 min and shocked the tail 2 min before the third test. On the first trial after the shock, both experimental groups had smaller siphon-withdrawal scores than the score of the control group. This difference was statistically significant for the 10-mA group [$t$ = 2.49 with 18 degrees of freedom (df); $P < 0.05$]. On subsequent trials, both experimental groups had larger withdrawal scores than the score of the control group. This difference was statistically significant for the 100-mA group 60 min after the shock ($t$ = 2.35 with 18 df, $P < .05$).

We next repeated these experiments with an intertrial interval of 2 min (rather than 15 min) to further explore the time course of inhibition and to facilitate subsequent physiological analysis. In addition, we gave either 2 or 10 pretest trials before the shock to investigate the effect of habituation. Thus, the animals with 10 pretests also served as no-shock controls over the first 10 trials. The animals with 2 pretests had smaller siphon-withdrawal scores than the control animals had on trials 3–5 (Fig. 1*B*). This difference was statistically significant on trial 4 ($t$ = 2.29 with 38 df; $P < 0.05$). On subsequent trials, the shocked animals had larger withdrawal scores than those of the control animals; this difference reached marginal statistical significance on trials 9 and 10 ($P < 0.05$; one-tail test in each case). By contrast, the animals with 10 pretests showed no inhibition following tail shock (trials 11–13). Despite the habituation produced by the pretests, these animals had significantly larger siphon-withdrawal scores on trial 12 than the animals with 2 pretests had on trial 4 ($t$ = 2.15; $P < 0.05$).

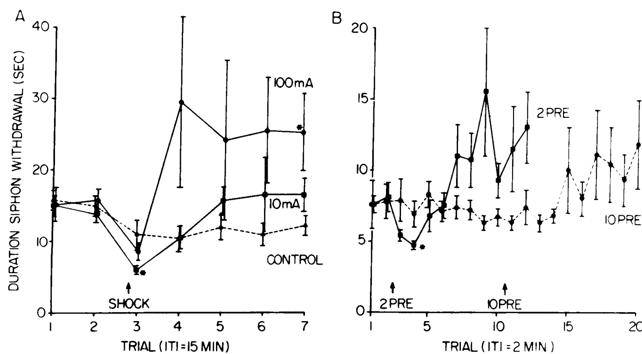


FIG. 1. Behavioral inhibition and sensitization of the siphon-withdrawal reflex. (*A*) The duration of siphon withdrawal in response to tactile stimulation of the siphon was measured once every 15 min. Two minutes before the third trial, a train of four shocks (60 Hz ac, 1.5-sec duration, 3-sec intershock interval) was delivered to the tail with capillary electrodes (arrow). Two different shock intensities [10 (■) and 100 (●) mA] were used. There was significant inhibition for the 10-mA group [compared to the no-shock control group (---)] on trial three and significant sensitization for the 100-mA group on trial seven ($n$ = 10 per group; * = $P < 0.05$). (*B*) As in *A* except that the intertrial interval (ITI) was 2 min. Also, there were either 2 (2 Pre) or 10 (10 Pre) pretest trials before the shock. The shock intensity was either 10 or 25 mA; results for the two intensities were similar and have been pooled. There was significant inhibition for the 2-pretest group (compared to the 10-pretest group) on trial 4 and marginally significant sensitization on trials 9 and 10. The 10-pretest group showed no inhibition after tail shock ($n$ = 20 per group). Vertical lines are SEMs.

The results of these two experiments show that (*i*) aversive stimulation (tail shock) can produce both inhibition and sensitization of the withdrawal reflex; (*ii*) the inhibition is transient and precedes the sensitization; (*iii*) the inhibition is produced by relatively weak intensities of shock, whereas the sensitization is more prominent with stronger intensities; and (*iv*) the inhibition is not observed after habituation training.

**Tail Shock Produces Inhibition of Monosynaptic Excitatory Postsynaptic Potentials (EPSPs) and Narrowing of Action Potentials in the Siphon Sensory Cells.** Sensitization of the siphon-withdrawal reflex is due in part to presynaptic facilitation of the connections that the siphon sensory cells make on their central target neurons (9, 10). To examine whether inhibition of the reflex might have an analogous cellular mechanism, we tested the effect of tail shock on the direct excitatory connections from siphon sensory cells to siphon motor neurons (9, 28). The amplitude of the monosynaptic EPSP from a sensory cell to a motor cell was significantly less on the trial immediately after the shock than on the trial before the shock (Fig. 2*A*; $t$ = 6.33 with 13 df; $P < 0.001$). This decrease in amplitude of the synaptic potential is probably not attributable to homosynaptic depression because it was

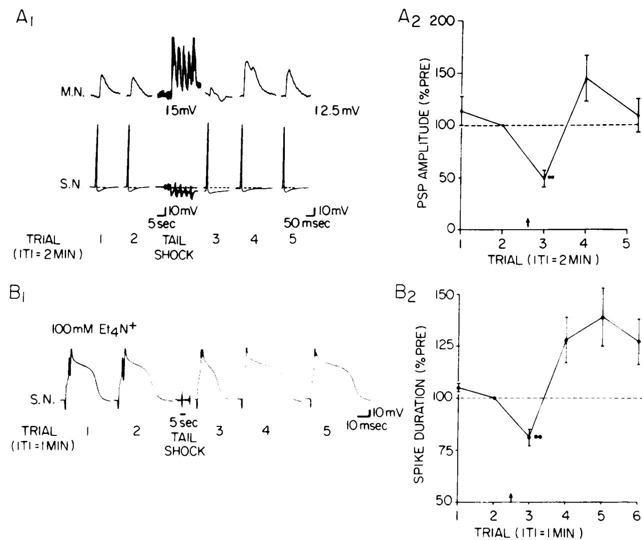


FIG. 2. Tail shock produces inhibition (*A*) and spike narrowing (*B*) in siphon sensory cells. ($A_1$). Example of inhibition and facilitation of monosynaptic EPSP by tail shock. The EPSP from a siphon sensory neuron (S.N.) to a siphon motor neuron (M.N.) was tested once every 2 min. Thirty seconds before the third trial, a train of four or five shocks (75 mA ac) was delivered to the tail. The EPSP was inhibited on trial 3 and facilitated on trials 4 and 5. Tail shock also produced hyperpolarization of the S.N. (see the dashed line on the S.N. trace). ($A_2$) Average results from 14 sensory cells in four preparations. EPSP (PSP) amplitudes are expressed as a percentage of their value on trial 2 (dashed line = 100%). Tail shock was delivered between trial 2 and 3 (arrow). There was significant inhibition of the EPSPs on trial 3 compared to that on trial 2 (** = $P < 0.01$) and marginally significant facilitation on trial 4 ($P < 0.05$ one-tailed test). ($B_1$) Example of spike narrowing and broadening in a S.N. produced by tail shock in the presence of 100 mM $Et_4N^+$. The duration of the action potential in the S.N. was tested once every 60 sec. Tail shock (100 mA) between trials 2 and 3 produced narrowing of the spike on trial 3 and broadening on trials 4 and 5. ($B_2$) Average results from 10 sensory cells in 10 preparations. There was significant narrowing of the action potential on trial 3 compared to that on trial 2 ($P < 0.01$) and significant broadening on trials 4–6 ($P < 0.05$ in each case).

significantly greater than the depression from trial 1 to 2 ($t$ = 4.07; $P < 0.01$). With continued testing, facilitation of the EPSP emerged, reaching a maximum ≈2.5 min after the shock. Thus, tail shock of appropriate intensity can produce first inhibition and then facilitation of the direct connections from sensory neurons to motor neurons.

Inhibition of EPSPs from sensory to motor cells could have either presynaptic or postsynaptic mechanisms. The observation that the shock also produces hyperpolarization of the sensory cells (Fig. 2$A_1$) suggested that the inhibition might be in part presynaptic. To examine this possibility further, we tested the effect of tail shock on the duration of the action potential in siphon sensory cells. Previous experiments have shown that the ionic mechanisms underlying presynaptic inhibition (a decrease in $Ca^{2+}$ current and an increase in the specific $K^+$ current designated S) produce narrowing of the action potential (25, 26, 29). To make it easier to detect changes in action-potential duration, we bathed the nervous system in seawater containing 100 mM tetraethylammonium ($Et_4N^+$) chloride, which increases the duration of the action potential by blocking the delayed and $Ca^{2+}$-dependent $K^+$ currents. The duration of the action potential in the sensory neuron was significantly less on the trial immediately after the shock than on the trial before the shock (Fig. 2*B*; $t$ = 5.13 with 9 df; $P < 0.01$). Moreover, this decrease was significantly greater than the decrease from trial 1 to trial 2 ($t$ = 3.07; $P < 0.05$). With continued testing, broadening of the action potential emerged, reaching a maximum ≈2.5 min after the shock.

**An Identified Phe-Met-Arg-Phe-$NH_2$-Positive Neuron Participates in Mediating the Inhibitory Effects of Tail Shock.** Like tail shock, Phe-Met-Arg-Phe-$NH_2$ produces inhibition, hyperpolarization, and narrowing of action potentials in the siphon sensory cells (14, 24, 26); these effects are illustrated in Fig. 3 *A* and *B*. In addition, Phe-Met-Arg-Phe-$NH_2$-positive fibers are present in the neuropil of the abdominal ganglion, the location of the sensory cells (43). Therefore, we initiated a search for Phe-Met-Arg-Phe-$NH_2$-positive neurons that might mediate the inhibitory effects of the shock. Using a combination of immunofluorescence and fluorescent dye backfilling from the abdominal ganglion, we located several candidate neurons in the other ganglia. We describe here one of them, a single neuron in the left pleural ganglion, which we believe is the only neuron in that ganglion that has both an axon in the pleural-abdominal connectives and positive Phe-Met-Arg-Phe-$NH_2$-like immunofluorescence (Fig. 3*C*). We have named this cell "LPL16," extending a previously established numbering system for pleural neurons (30).

To examine whether this identified neuron participates in mediating the inhibitory effects of tail shock, we first determined whether intracellular stimulation of the neuron produces inhibition of EPSPs from siphon sensory cells to motor cells. In eight of nine preparations, stimulation of LPL16 caused substantial inhibition of the EPSP within one to two trials (Fig. 4*A*). The inhibition was typically short lived, wearing off within one trial after firing of LPL16 stopped.

Since tail shock and Phe-Met-Arg-Phe-$NH_2$ both produced inhibition in part by narrowing the action potential in the sensory neuron, we next examined whether stimulating LPL16 also narrows the action potential in the presence of 100 mM $Et_4N^+$. In three of three preparations, intracellular stimulation of LPL16 produced noticeable narrowing of the action potential in the sensory neuron (Fig. 4*B*).

Stimulation of LPL16 thus qualitatively simulates both tail shock and exogenous Phe-Met-Arg-Phe-$NH_2$ in producing inhibition and narrowing of action potentials in the sensory neurons. We noted one discrepancy, however: stimulation of LPL16 produced no detectable hyperpolarization of the sensory neuron (Fig. 4*A*). This result might be explained if the synapses of LPL16 were located on remote processes (rather than the cell body) of the sensory neuron. Alternatively, since the Phe-Met-Arg-Phe-$NH_2$ antibody is not completely specific, the transmitter of LPL16 might be a related peptide and not Phe-Met-Arg-Phe-$NH_2$ itself.

If LPL16 participates in mediating the inhibitory effects of tail shock, it should also be excited by the shock. Fig. 4*C*

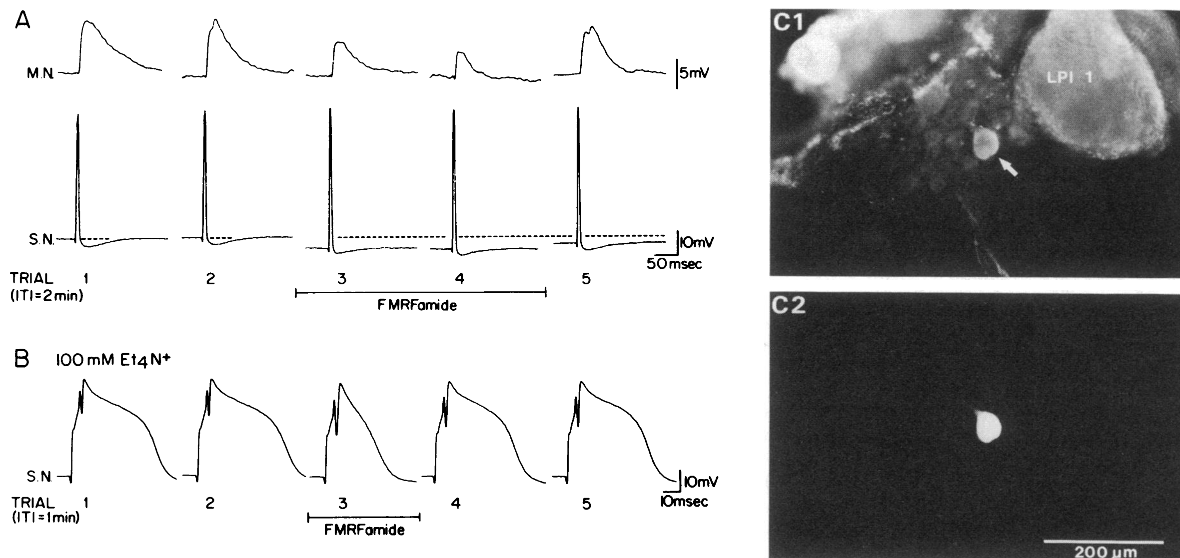


FIG. 3. Phe-Met-Arg-Phe-$NH_2$ (FMRFamide) produces inhibition and spike narrowing in siphon sensory cells. (*A*) Example of inhibition of monosynaptic EPSP from a siphon sensory neuron (S.N.) to a siphon motor neuron (M.N.) during bath application of 10 μM Phe-Met-Arg-Phe-$NH_2$ (trials 3 and 4) and recovery following washout (trial 5). Phe-Met-Arg-Phe-$NH_2$ also produced hyperpolarization of the S.N. (see the dashed line on the S.N. trace). ITI was 2 min. (*B*) Example of spike narrowing in a S.N. in 100 mM $Et_4N^+$ during application of 10 μM Phe-Met-Arg-Phe-$NH_2$ (trial 3) and recovery following washout (trials 4 and 5). ITI was 1 min. (*C*) Phe-Met-Arg-Phe-$NH_2$ immunofluorescence in the left pleural ganglion. (*C1*) The dorsal surface of the ganglion viewed with rhodamine filters showing Phe-Met-Arg-Phe-$NH_2$ immunofluorescence. LPL16 (arrow), the pleural giant neuron (LPL1), and several other cells (upper left corner) have positive immunofluorescence. (C2) Same field as *C1* viewed with Lucifer Yellow filters. LPL16 was injected with Lucifer Yellow by iontophoresis (1-nA hyperpolarizing pulses of 500-msec duration at 1 Hz for 1 hr).

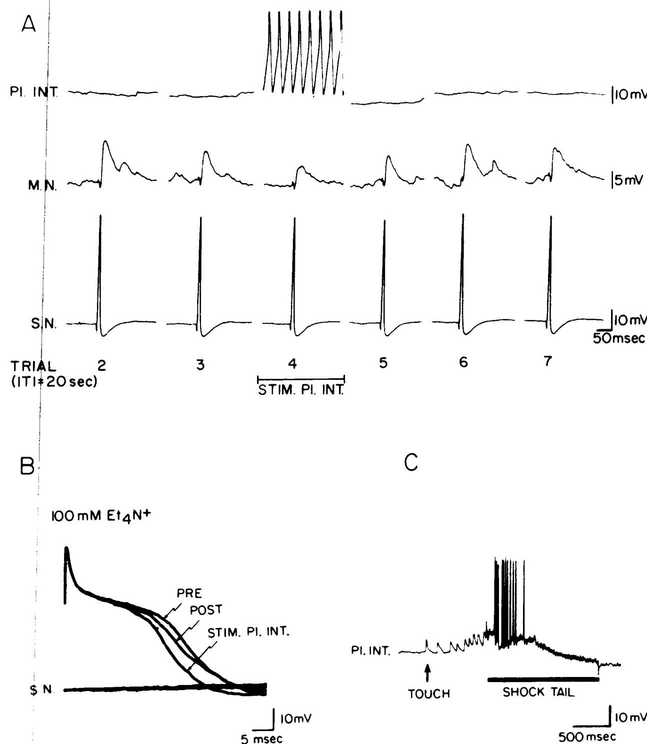


FIG. 4. Stimulation of LPL16 produces inhibition and spike narrowing in siphon sensory cells. (*A*) Example of inhibition produced by stimulating LPL16. The EPSP from a sensory neuron (S.N.) to a motor neuron (M.N.) was tested once every 20 sec. One second before the fourth trial, LPL16 pleural interneuron (Pl. Int.) was stimulated with intracellular current, causing it to fire at 30 Hz for 3 sec (STIM. Pl. Int.). The EPSP was inhibited on the first trial after the start of LPL16 stimulation. (*B*) Example of spike narrowing produced by stimulating LPL16 in the presence of 100 mM $Et_4N^+$. The spike in a sensory neuron is shown before (PRE), during (STIM. Pl. INT.), and after (POST) intracellular stimulation of LPL16. (*C*) Example of the response of LPL16 to touching and then shocking the tail with a capillary electrode (75 mA ac).

shows firing of LPL16 produced by tail shock in a semi-intact preparation. These qualitative results suggest that LPL16 does participate in mediating behavioral inhibition. We did not test whether hyperpolarizing LPL16 (preventing it from firing) reduces the inhibition produced by tail shock. However, we doubt that this neuron by itself can account quantitatively for all of the inhibitory effects of tail shock, since the effects produced by firing LPL16 are generally smaller and shorter lasting than those produced by tail shock, despite the fact that we have usually fired LPL16 more than it fires in response to tail shock.

## DISCUSSION

**The Relationship Between Inhibition and Sensitization.** Our results indicate that, as in vertebrates, aversive stimuli not only sensitize but also inhibit the *Aplysia* siphon-withdrawal reflex. These opposite modulatory effects of tail shock can be dissociated behaviorally in several ways. First, the inhibition is much briefer and precedes the sensitization. This temporal offset of the two effects might be explained by sequential activation of the two modulatory pathways. However, we think it more likely that the two modulatory effects are activated simultaneously, with the inhibitory effect being stronger initially and decaying more rapidly. In support of this possibility, our cellular data indicate that tail shock immediately activates both inhibitory and facilitatory interneurons (Fig. 4*C*; refs. 13 and 17). Furthermore, Phe-Met-Arg-Phe-$NH_2$ can transiently override the effect of serotonin when they are applied simultaneously (24). Second, the inhibition is activated by relatively weak stimuli, whereas sensitization is more prominent with stronger stimuli (Fig. 1*A*). Finally, we have confirmed the report of Carew and colleagues (20, 21) that inhibition is not observed when the reflex is habituated (Fig. 1*B*). These three factors may explain why inhibition of the reflex has not been detected previously, since most previous experiments have been carried out in ways that favor the appearance of sensitization.

**Physiological Mechanisms of Behavioral Inhibition.** The fact that tail shock produces inhibition and spike narrowing in siphon sensory neurons suggests that these physiological effects contribute to the behavioral inhibition. Additional mechanisms are also likely to contribute, however, since siphon withdrawal is significantly depressed for at least 2 min after tail shock, whereas the inhibition and narrowing of action potentials produced by tail shock both persist for <2 min. Therefore, it is likely that noxious stimuli also have inhibitory actions at sites other than the sensory neurons (i.e., the interneurons or motor neurons), just as tail shock has facilitatory effects at other sites in addition to the sensory neurons (28, 31). Some of these inhibitory actions may be mediated by an identified interneuron in the abdominal ganglion, L16, which is activated by connective shock and which inhibits excitatory interneurons in the circuit for the gill- and siphon-withdrawal reflex (12).

**Transmitters Involved in Inhibition.** Our cellular data indicate that tail shocks produce their inhibitory effects by hyperpolarizing the siphon sensory neurons, narrowing the sensory neuron action potentials, and inhibiting synaptic transmission from sensory neurons to their followers. All of these actions are consistent with the known actions of Phe-Met-Arg-Phe-$NH_2$ (14, 24, 26). The identification of an inhibitory interneuron (LPL16) that contains Phe-Met-Arg-Phe-$NH_2$ immunoreactivity and is activated by stimulation of the tail supports the involvement of Phe-Met-Arg-Phe-$NH_2$ in the inhibition produced by tail shock. However, because the anti-Phe-Met-Arg-Phe-$NH_2$ antibody is not completely specific, direct biochemical evidence will be necessary before we can make this transmitter attribution with confidence. Moreover, our evidence does not exclude a role for other inhibitory transmitters. Both dopamine and arginine vasotocin have also been shown to produce presynaptic inhibition of siphon sensory cells (14, 22, 23). Thus, just as several transmitters (serotonin, small cardioactive peptide or SCP, and the unknown transmitter of L29) may contribute to behavioral sensitization by presynaptically facilitating siphon sensory neurons (12–17), so may the behavioral inhibition described here be mediated by several different transmitters (Fig. 5).

**Possible Behavioral Roles of the Inhibitory Process.** The inhibition produced by tail shock could play several functional roles in the animal's behavior. First, it might serve transiently to suppress reflex behaviors that interfere with more important defensive behaviors, such as escape locomotion. In so doing, it would elevate the threshold and delay the onset of sensitization. Kupfermann and Weiss (32) have noted similar effects on feeding, which is inhibited immediately after tail pinch and facilitated 4 min later. These effects may also be analogous to ones seen in vertebrates, where painful stimulation can lead to the release of enkephalins (peptides that are remote homologues of Phe-Met-Arg-Phe-$NH_2$) that act presynaptically to inhibit primary sensory neurons, presumably suppressing reflex behaviors (33, 34).

Second, the inhibition could play a role in conditioning of gill and siphon withdrawal (6–8). A feature of conditioning in both vertebrates (1, 2) and invertebrates (35) is that the same unconditioned stimulus (US) may serve either to facilitate or inhibit a given response depending upon whether or not the conditioned stimulus (CS) predicts the occurrence of the US. In situations where the CS predicts that the US will not occur,

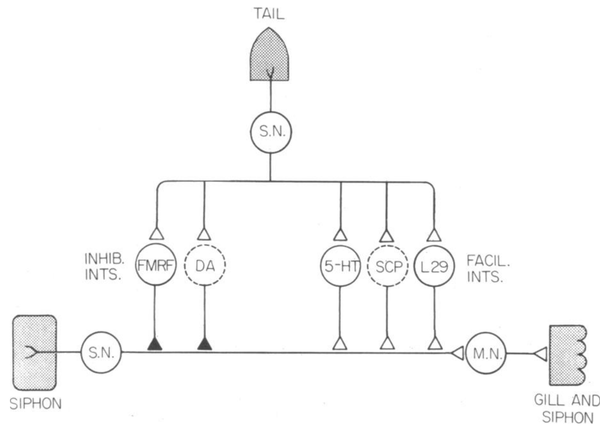


FIG. 5. Summary diagram of transmitters and identified neurons that modulate synaptic transmission from the siphon sensory cells. △, Facilitatory synapses; ▲, inhibitory synapses; solid circles, identified neurons; dashed circles, still-unidentified neurons that mediate the transmitter effect; FMRF, Phe-Met-Arg-Phe-$NH_2$; DA, dopamine; 5-HT, serotonin; SCP, small cardioactive peptide. The transmitter of L29 is not yet known.

the response to the CS may be inhibited. We have not yet tested such conditioned inhibition in *Aplysia*. However, we have demonstrated that adding unpredicted USs decreases excitatory conditioning (8). Although other explanations are possible (36), this effect could have the same underlying mechanism as conditioned inhibition (37). Our present results indicate that *Aplysia* possesses a neural mechanism that might mediate conditioned inhibition.

The presynaptic inhibitory pathway may also play a role in the temporal specificity of classical conditioning. No conditioning is seen if the US precedes the CS by 0.5 sec or more—that is, there is no "backward" conditioning (8). This result might be explained by the spike narrowing that tail shock produces in sensory neurons (Fig. 2*C*). Thus, if tail shock (the US) immediately precedes spike activity in the sensory neurons (the CS), it would be expected to reduce the $Ca^{2+}$ influx, which is thought to play a critical role in the activity-dependent enhancement of presynaptic facilitation that contributes to classical conditioning (refs. 11 and 38; see ref. 39 for a similar suggestion regarding pleural sensory neurons). Finally, LPL16 may also be activated by other events that cause inhibition of the siphon-withdrawal reflex, such as feeding and copulation (40–42).

Irrespective of the precise behavioral role of the inhibition, our results illustrate that environmental contingencies can act on the sensory neurons through two different modulatory systems, one facilitatory and one inhibitory. These modulatory systems utilize two different families of transmitters (including serotonin for facilitation and Phe-Met-Arg-Phe-$NH_2$ for inhibition) and engage two different second-messenger systems within the sensory neurons—the cAMP cascade for facilitation and the lipoxygenase pathway of arachidonic acid for inhibition. Therefore, our results suggest that behavioral events are represented within the nervous system by the activation of antagonistic modulatory systems and are rerepresented within single sensory neurons by the balance of actions of competing second-messenger systems. Thus, these results begin to reveal an unexpected richness at the cellular and molecular levels underlying the internal representation of external events.

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