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25. Abbreviations for the amino acid residues are A, Ala; D, Asp; E, Glu; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; P, Pro; Q, Gln; R, Arg; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Activity-Dependent Enhancement of Presynaptic Inhibition in *Aplysia* Sensory Neurons

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Tail shock produces transient presynaptic inhibition and longer lasting presynaptic facilitation of the siphon sensory neurons in *Aplysia*. The facilitation undergoes activity-dependent enhancement that is thought to contribute to classical conditioning of the gill- and siphon-withdrawal reflex. Inhibition of the sensory neurons has now also been shown to undergo activity-dependent enhancement when action potential activity in the sensory neurons is paired with inhibitory transmitter. This effect appears to involve an amplification of the same cellular mechanisms that are involved in normal presynaptic inhibition. These results suggest that activity-dependent enhancement may be a general type of associative cellular mechanism.

SEVERAL DIFFERENT ASSOCIATIVE cellular mechanisms may underlie aspects of learning, including activity-dependent enhancement of facilitation (*1*), associative changes in excitability (*2, 3*), long-term potentiation (*4*), and long-term depression (*5*). Activity-dependent facilitation is thought to contribute to classical conditioning of the gill- and siphon-withdrawal reflex in *Aplysia* (*6, 7*). This mechanism appears to be an amplification of a cellular mechanism of sensitization, that is, presynaptic facilitation of the siphon sensory neurons by serotonin acting through adenosine 3′,5′-monophosphate (cAMP) (*8*). We wished to examine whether activity-dependent enhancement is a more general phenomenon which occurs for other modulatory effects. An opportunity to test this idea was provided by the finding that, in addition to sensitization and classical conditioning, the gill- and siphon-withdrawal reflex also undergoes transient behavioral inhibition (*9, 10*). The inhibition is thought to be due in part to presynaptic inhibition of the siphon sensory neurons by the peptide FMRFamide acting through the arachidonic acid cascade (*10, 11*). We now report that, like facilitation, inhibition of the sensory neurons undergoes an associative, activity-dependent enhancement when spike activity in the sensory neurons is paired with inhibitory transmitter.

To test whether inhibition of the siphon sensory neurons is modulated by paired action potential activity, we puffed FMRFamide onto the cell body of a sensory neuron either paired or unpaired with a brief train of action potentials in the neuron (Fig. 1A) (*12*). In the first set of experiments, we examined the effect of this training on the amplitude of the synaptic connection from the sensory neuron to a motor neuron. Unpaired training produced rather short-lived inhibition of the excitatory postsynaptic potential (EPSP), whereas paired training produced longer lasting inhibition (Fig. 1B). On average, the two training procedures produced similar inhibition 1 min after training, but whereas the EPSP then returned to near baseline 2 min after unpaired training, it remained inhibited for several minutes after paired training (Fig. 1C). There was a significant overall effect of training procedure [$F(1,19) = 6.72$, $P < 0.02$] and significant differences in planned comparisons between paired and unpaired training 2 min [$F(1,57) = 9.57$, $P < 0.01$] and 3 min [$F(1,57) = 4.52$, $P < 0.05$] after training (*13*).

These results suggest that action potential activity paired with FMRFamide ("paired action potential activity") enhances the FMRFamide-induced inhibition. However, because high-frequency action potential trains can produce posttetanic potentiation (PTP) at these synapses (*14*), an alternative interpretation is that FMRFamide paired with an action potential train ("paired FMRF") decreases the potentiation produced by the train. If the paired and unpaired training procedures both produce a mixture of inhibition (caused by FMRFamide) and potentiation (caused by the action potential train), a difference between the training procedures could be due to a difference in either process. To address this issue, we first determined whether the action potential train we used (five spikes at 10 Hz) produced potentiation. Toward this end, we ran a second set of experiments with a similar design but with two new training procedures: one with the action potential train alone and one in which the EPSP was simply tested at the usual times. Testing produced synaptic depression that continued at a rate similar to that seen before training (Fig. 1, C and D). Compared to this control procedure, the train of action potentials produced potentiation that was modest (approximately 120% of control on each trial after training) but statistically significant [$F(1,19) = 4.61$, $P < 0.05$].

To analyze if paired action potential activity enhances inhibition or if paired FMRFamide reduces potentiation, we ran a third set of experiments comparing the effects of paired training and training with the FMRFamide puff alone (Fig. 1D). Paired training produced significantly greater inhibition than FMRFamide-alone training [$F(1,19) = 4.83$, $P < 0.05$]. This result cannot be explained by reduction of potentiation by paired FMRFamide, because the FMRFamide-alone procedure includes no action potential train and thus no potentiation. Rather, the data provide support for the idea that paired training enhances inhibition. It is possible that unpaired training also modifies the effects of action potential activity or FMRFamide.

We next tested if the activity-dependent enhancement of inhibition by paired action potential activity occurred pre- or postsynaptically. We first tested a possible postsynaptic mechanism, a pairing-specific change in input resistance in the motor cell. To

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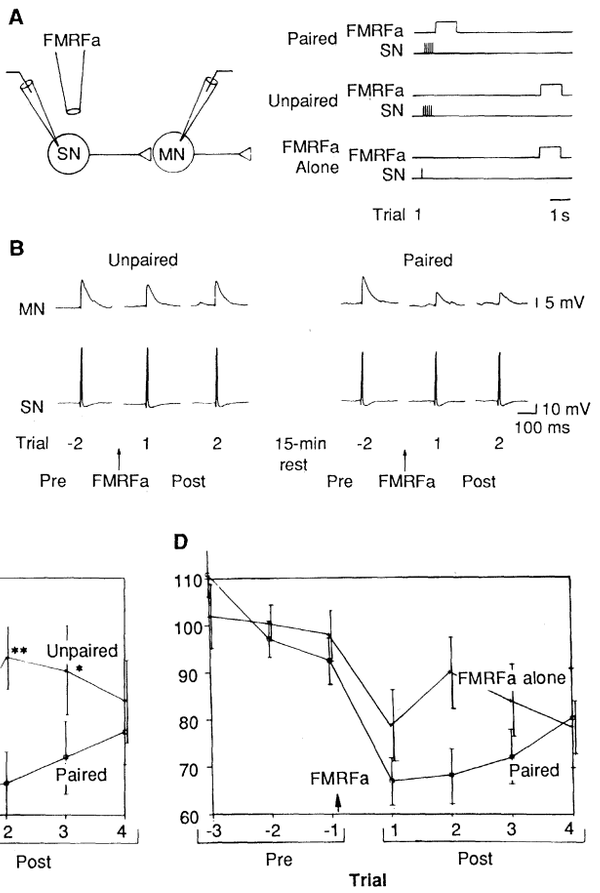

Fig. 1. Activity-dependent enhancement of inhibition. (**A**) Experimental procedure. (Left) Physical arrangement. During testing, an intracellular depolarizing current pulse elicited a single action potential in a sensory neuron (SN), producing an EPSP in a motor neuron (MN) with an intertrial interval (ITI) of 1 min. Training occurred immediately after the fourth or fifth test trial. (Right) Training procedures. A brief train of action potentials (five spikes at 10 Hz) was elicited in the sensory cell either 0.5 s (paired) or 5.5 s (unpaired) before FMRFamide (FMRFa) was pressure-ejected onto the cell. The first action potential of the train was the last pretest (trial 1). In other experiments, FMRFamide was delivered without any action potential train in the sensory neuron (FMRFamide alone). (**B**) Results from a representative experiment. Since it was impossible to apply the FMRFamide equally to two different cells, both the paired and unpaired procedures were performed on the same sensory cell with a 15-min period of no stimulation between them. The order of the training procedures was counterbalanced across experiments. In this experiment, the cell first received unpaired training, which produced relatively short-lived inhibition of the EPSP from the sensory cell to the motor cell. By contrast, paired training produced larger and longer lasting inhibition. ITI, 1 min. (**C**) Average results from 20 experiments, comparing the effects of paired and unpaired training. One experiment was performed per animal. The amplitude of the EPSP has been normalized to the average value on the last three trials before training (pre). Pretest values were approximately equal for the paired and unpaired conditions (7.3 ± 1.2 mV and 6.7 ± 0.8 mV, respectively). Training occurred at the time indicated by the arrow. Paired training produced greater inhibition than unpaired training on the subsequent trials (post). Error bars, SEM. $*P < 0.05$. $**P < 0.01$. ITI = 1 min. (**D**) Average results from 20 experiments, comparing the effects of paired and FMRFamide-alone training. Pretest values were approximately equal for the paired and FMRFamide-alone conditions (6.1 ± 0.9 mV and 5.5 ± 1.0 mV, respectively). Paired training produced greater inhibition than FMRFamide-alone training. ITI, 1 min.

measure input resistance, we injected brief hyperpolarizing current pulses into the soma of the motor cell 5 s before each EPSP, in 8 of the 20 experiments included in Fig. 1C. The amplitude of the hyperpolarization in the motor cell did not change after either paired or unpaired training, nor was there a difference between the two training procedures (Fig. 2A) [$F(1,7) = 0.49$, not significant]. These results demonstrate that a change in input resistance measured in the soma cannot account for the effect of pairing shown in Fig. 1C. However, changes in input resistance in distant processes or changes in other postsynaptic factors, such as receptor sensitivity, are not ruled out.

Since FMRFamide acts presynaptically by opening K^+ channels and closing Ca^{2+} channels, thus reducing Ca^{2+} influx during action potentials (*15*), we decided to examine whether these presynaptic effects are enhanced by paired action potential activity. To address this question, we ran another set of experiments comparing the effects of paired and unpaired training on the duration of the action potential in the sensory neuron in the presence of 100 m*M* tetraethylammonium (TEA) (*16*). TEA blocks part of the K^+ current, prolonging the duration of the action potential and thus making Ca^{2+} the major inward charge carrier. The duration of the action potential in TEA solution is therefore a sensitive assay for changes in either the remaining K^+ current or the Ca^{2+} current. The results of these experiments were similar to the results of the EPSP experiments (Fig. 2B). Paired and unpaired training produced approximately equal narrowing of the action potential 1 min after training, but the effect lasted longer after paired training. There was a significant overall effect of training procedure [$F(1,19) = 3.91$, $P < 0.05$, one-tailed] and significant differences in planned comparisons between paired and unpaired training 2 min [$F(1,57) = 7.07$, $P < 0.01$] and 3 min [$F(1,57) = 7.00$, $P < 0.02$] after training. These results suggest that paired action potentials enhance the same cellular mechanisms that are thought to be involved in normal presynaptic inhibition. We do not yet know whether there is a pairing-specific increase in K^+ current, a decrease in Ca^{2+} current, or both. We also do not know whether these biophysical changes in the sensory neurons contribute to activity-dependent inhibition under physiological conditions, that is, in the absence of TEA; our results merely indicate that they could.

We do not yet have any information regarding the molecular mechanism of activity-dependent inhibition. Activity-dependent facilitation appears to involve an enhancement of the same cellular and molecular mechanisms that are involved in normal presynaptic facilitation. Specifically, Ca^{2+} influx during the action potentials appears to enhance the production of cAMP by facilitatory transmitter, leading to enhanced depression of K^+ current and enhanced transmitter release (*8*). Since normal presynaptic inhibition is mediated by the lipoxygenase pathway of arachidonic acid (*11*), activity-dependent inhibition may similarly involve an enhancement of the arachidonic acid cascade by Ca^{2+} influx during action potentials (*17*). Hippocampal long-term potentiation may also involve arachidonic acid, possibly as a transsynaptic messenger molecule (*11, 18*). If so, activity dependence of the arachidonic acid cascade could contribute to the associative properties of that effect as well (*4*).

We also do not yet have a clear idea of the behavioral role of the inhibition or of its activity dependence in *Aplysia*. Because tail shock produces both inhibition and facilitation, the two effects tend to cancel each other out, although the inhibition has a shorter duration (*9, 10*). Having two opposing modulatory processes may be a way of achieving finer control of the modulation or of delaying the expression of the facilitation. In either case, the activity dependence of inhibition may partially offset the activity dependence of facilitation during classical conditioning. Activity-dependent inhibition might also play a role in a variety of other

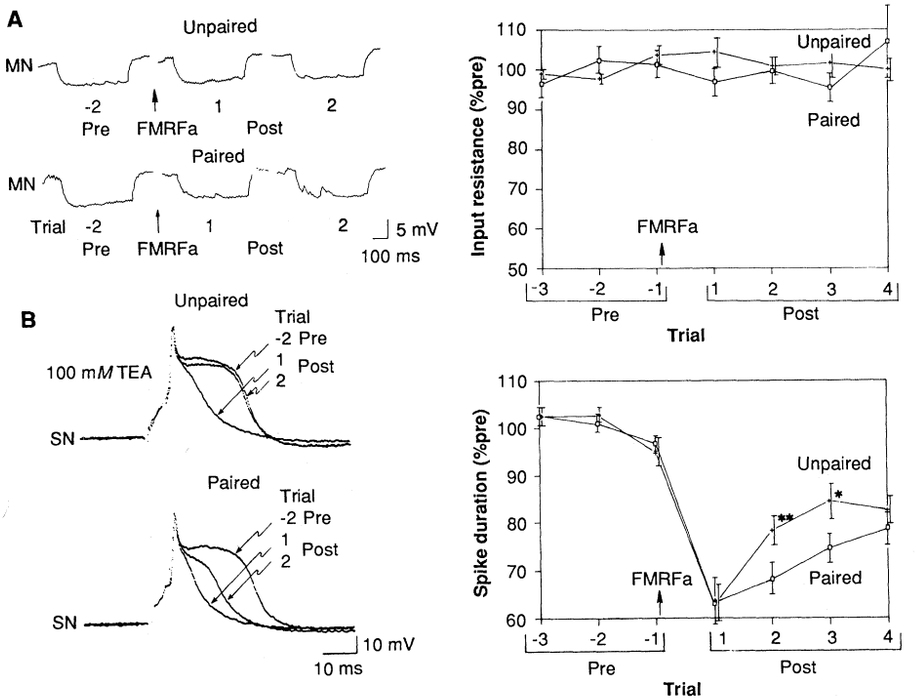

Fig. 2. Tests of post- and presynaptic mechanisms contributing to activity-dependent inhibition. (**A**) Tests of changes in input resistance in the motor neuron. In eight of the experiments in Fig. 1C, a 500-ms constant current hyperpolarizing pulse was injected into the motor neuron 5 s before each test of the EPSP. The amplitude of the hyperpolarization at the end of the pulse was measured with a Wheatstone bridge circuit, giving an estimate of postsynaptic input resistance. (Left) Results from a representative experiment (the same experiment as in Fig. 1B). (Right) Average results from the eight experiments. Pretest values were approximately equal for the paired and unpaired conditions (5.1 ± 0.3 mV and 5.0 ± 0.4 mV, respectively). Neither paired nor unpaired training produced any consistent change in the input resistance of the motor neuron. ITI, 1 min. (**B**) Tests of changes in the duration of the action potential in the sensory neuron in 100 m*M* TEA solution. (Left) Results from a representative experiment. The action potential on the second trial before training (−2 Pre) and the first and second trials after training (1 and 2 Post) have been superimposed to facilitate comparison of their durations. (Right) Average results from 20 experiments comparing the effects of paired and unpaired training. Pretest values were approximately equal for the paired and unpaired conditions (23.8 ± 2.8 ms and 22.7 ± 2.9 ms, respectively). Paired training produced greater narrowing of the action potential in the sensory neuron than unpaired training. ITI, 1 min.

learning phenomena observed in *Aplysia* gill and siphon withdrawal, including habituation, contingency learning, and aversive operant conditioning (*7, 19*).

Activity-dependent enhancement of inhibition is an associative cellular mechanism in individual neurons that detects and records that two events have occurred in close temporal contiguity. The discovery that activity-dependent enhancement occurs for inhibition as well as facilitation in *Aplysia* sensory neurons indicates that this type of mechanism is not limited to a single direction of action, transmitter, or second messenger system. Facilitation of the sensory neurons is produced by serotonin acting through cAMP (*20*), and inhibition is produced by FMRFamide, probably acting through arachidonic acid (*10, 11*). Activity-dependent enhancement has also been reported with acetylcholine and guanosine 3′,5′-monophosphate (cGMP) in cat motor cortex (*3*) and with octopamine at the crayfish neuromuscular junction (*21*). These results suggest that activity-dependent enhancement of modulatory effects may be a general type of associative mechanism and might also occur for modulation involving other transmitters and second messengers.

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13. Normalized data were analyzed with a two-way analysis of variance with one repeated measure (training procedure was one factor and test trial was the second factor). If there was a significant overall effect of training procedure, planned comparisons between procedures were made at each individual test trial to assess the time course of the effect [W. L. Hays, *Statistics* (Holt, Rinehart & Winston, New York, 1963)]. Statistical comparisons of training procedures were all "within cell," because there were substantial differences between cells in, for example, their rates of synaptic decrement and their responses to FMRFamide.
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Directed Movement of Latex Particles in the Gynoecia of Three Species of Flowering Plants

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The secretory matrix of the stylar-transmitting tract of angiosperms has been characterized as a nutrient medium for the growth of pollen tubes, acting to guide tubes to the ovules. When nonliving particles (latex beads) were artificially introduced onto the transmitting tracts of styles of *Hemerocallis flava*, *Raphanus raphanistrum*, and *Vicia faba*, they were translocated to the ovary at rates similar to those of pollen tubes. Direct observations were made on the movement of individual beads along the secretory epidermis in the style and ovary of *Vicia faba*. The transmitting tract may play an active role in extending tube tips to their destination in the ovary.

POLLINATION INVOLVES THE PROcesses of pollen capture by the stigma, hydration, and pollen tube growth through the gynoecial tissues to place the sperm cells they carry in the embryo sac in the ovule. The pollen tube grows by tip extension, restricting the constant amount of cytoplasm there by production of a callose wall or plug that periodically sequesters the living part of the cell at the tube tip. A mechanism for tip growth in pollen has been proposed that accounts for the clearly demonstrated capability of pollen tube growth in vitro (*1*). In this model, internal osmotic pressure is the force behind tip extension; however, in vitro pollen tubes typically do not grow as fast or as long as they do in vivo.

Beginning just below the surface cells of the stigma, an extracellular matrix of secretions defines the path of pollen tube growth to the ovule in the ovary (*2*). In open styles, the matrix covers the surface of the inner epidermal cells, and in closed styles, the matrix occurs in the intercellular spaces of the transmitting tract. Pollen tube growth is typically restricted to this secretory matrix. Attempts to characterize the chemical components of the stylar medium have revealed a variety of substances, with high molecular weight compounds being polysaccharide mucilages (pectic compounds and proteoglycans) and complex proteins (glycoproteins and lipoproteins) (*3, 4*). Arabinogalactans appear frequently in stigma and stylar secretions, and it is assumed that they have a role in at least adhesion of the pollen grain to the stigma (*5*).

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To our knowledge, a role has not been proposed for the stylar matrix in the pollination process other than one of a nutritional fluid and, hence, a pollen tube guide. Here, we examine the possibility that the stylar matrix may play an active role in facilitating directed pollen tube growth. We applied inert particles the size of a pollen tube tip to the transmitting tracts of three species. The growth of pollen tubes and the movement of latex beads were followed in separate styles.

In *Vicia faba*, emasculations 2 days before anthesis were necessary to avoid self-pollination; the other two species are self-incompatible. Pollinations of *Hemerocallis flava* were done in situ. In this variety, growth of pollen tubes to the ovary is rare, so experiments were done on the upper half of the 120-mm-long style. Flowers of *Raphanus raphanistrum* and *V. faba* were excised and pedicels were placed in a 2% agar medium; the flowers were hand-pollinated and placed under lights. Red-dyed monodispersed latex polystyrene beads (2.5% solid latex, Polysciences) with a mean diameter of 6 μm were applied from a syringe in a 2-μl drop containing thousands of beads. In *H. flava*, beads were applied to the stigma directly, in situ. For both *R. raphanistrum* and *V. faba*, flowers were placed in vitro for bead applications, as done for the pollinations. In both, the stigma and part of the style were excised (Fig. 1, B and C) before bead application, because direct application to the stigma resulted in no bead movement into the style.

Pollinated samples from all species were fixed, at various times after pollination, from 2.5 to 25 hours, in a 3 to 1 mixture of ethanol to glacial acetic acid and then left overnight. Tissue was then washed in 70% ethanol for 20 minutes and softened in 1*M* NaOH at 70°F. The samples were squashed in aniline blue and viewed on a Zeiss fluorescence microscope, equipped with Zeiss filter set 487705. Many pollen tubes grow initially on the stigma, but only a subset of these reach the ovary to effect fertilization. The distance that the longest pollen tubes had traveled was measured with an ocular micrometer to get an accurate measure of the pollen tube growth rates that effect fertilization. In *H. flava*, the red beads could be seen through the semitransparent tissue of the style moving in a front that was composed of hundreds of beads. Measurements of this movement were taken in situ approximately every hour. At various times (2 to 10 hours) after bead application in *R. raphanistrum* and *V. faba*, gynoecia were wiped clean with a tissue to remove any beads on the outer surface and dissected to expose the transmitting tract. The samples were placed in a depression slide containing 2% agar and were viewed under a light microscope. Only a small subset of the applied beads would be seen on the transmitting tract at this time. The distance the farthest beads had traveled was measured with an ocular micrometer. Less than 20 beads were translocated to the region of the

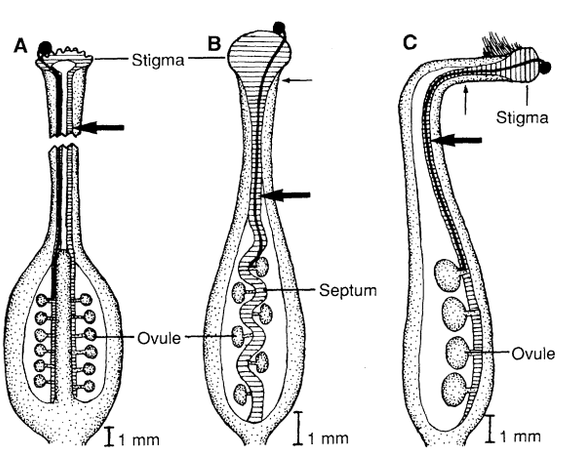

Fig. 1. Diagram of longitudinal sections of gynoecia, showing the path pollen tubes travel in compatible pollinations of (**A**) *H. flava*, (**B**) *R. raphanistrum*, and (**C**) *V. faba*. The transmitting tract is represented by cross-hatching. Large arrows indicate areas of tract shown in Fig. 2, A, C, and E. Small arrows in (B) and (C) indicate where the gynoecia were cut for bead applications.