Facilitation at neuromuscular junctions: Contribution to habituation and dishabituation of the Aplysia gill withdrawal reflex

(synaptic plasticity/behavioral plasticity)

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ABSTRACT The gill withdrawal reflex of Aplysia has been used as a model for studying the neuronal mechanisms of habituation, a behavioral plasticity. We have assessed the contribution of neuromuscular facilitation, an elementary synaptic plasticity, during habituation of the reflex by recording gill muscle potentials, which we show are caused by excitatory junctional potentials. These potentials show systematic frequency-dependent changes in amplitude. The gill withdrawal evoked by central motor neuron firing during each habituation trial is determined by facilitation of the excitatory junctional potentials during the trial and the facilitated state of the initial excitatory junctional potential in a trial, determined by neuron activity prior to the trial. The neuromuscular junctions, therefore, act like a frequency-dependent amplifier of central motor activity. They are fully responsive to the dynamic changes of motor neuron firing that occurs during habituation and especially after dishabituation.

The neural correlates of habituation have been extensively studied in three invertebrate preparations, the crayfish (Procambarus) (1-3), the locust (Schistocerca) (4), and the sea hare (Aplysia). Habituation, the decrement of a behavioral response with repeated stimulation and its restoration or dishabituation by a novel stimulus, can be studied in these preparations at the level of synaptic communication between neurons. In these animals, and possibly in vertebrates (5), homosynaptic depression at central neuronal synapses has been identified as a mechanism of habituation (1-4, 6, 7).

In Aplysia the reflex withdrawal of the gill evoked by siphon stimulation has been used as a model for the study of habituation (7-9). Several central motor neurons participate in this reflex, and studies of motor neuron L7 have shown that it receives excitatory postsynaptic potential (EPSP) input from a cluster of central sensory neurons (10-12). These EPSPs decrease in amplitude with successive stimuli and therefore, as a consequence of a decrease in the number of quanta of transmitter released (13) at the sensory-motor chemical synapses, homosynaptic depression has been proposed as the mechanism of habituation of the reflex (7, 9). The train of action potentials evoked by synaptic input to neuron L7 during behavioral habituation and dishabituation has been shown (9) but the firing of other major motor neurons (LDG1 and LDG2) has not. The firing of L7 decreases slowly during behavioral habituation of the gill and recovers slowly after dishabituation (9). If habituation is caused solely by homosynaptic depression at sensory-motor synapses (9, 13), the firing of the motor neurons should parallel the evoked gill withdrawal because these neurons synapse directly on gill muscles and are reported to produce temporally stable nondecrementing gill withdrawal (10, 14). However, it was shown that the neuron-gill muscle synapses showed pronounced facilitation (14).

We examined the possibility that changes in the efficacy of gill neuromuscular junctions contributed to habituation by recording discrete gill potentials evoked by the firing of central motor neurons. The amplitude of these potentials changed systematically with the firing frequency of motor neurons, suggesting that gill potentials reflect the facilitation of gill muscle excitatory junctional potentials (EJPs). This was confirmed by intracellular recording from muscles during the selective activation of gill motor neurons. Further experiments showed that frequency-dependent homosynaptic facilitation of EJPs has a powerful effect on gill withdrawal during behavioral habituation and dishabituation in addition to central synaptic mechanisms.

METHODS

Aplysia californica obtained from Pacific Bio-Marine, weighing 100–300 g, were used. They were kept in tanks of Instant Ocean at 15° with a 13:11 cycle of light and dark before use. For habituation experiments, more than 30 animals were reduced to a preparation of the siphon-mantle-gill with the parietovisceral ganglion (PVG) (abdominal ganglion) (9), attached by the siphon, branchial, and genital-ctenidial nerves (15). Each preparation was pinned out in a chamber filled with Instant Ocean maintained at 15°. Tactile stimulation of the siphon with a solenoid-driven stylus (2 mm diameter) evoked the gill withdrawal reflex. The solenoid was activated by pulses (30 msec duration) from a Grass stimulator, adjusted in voltage to produce a force equivalent to a 2- or 5-g weight measured on the Grass transducer, equal to the “weak-moderate” force used by others (10). The activity of neurons (L7, LDG1, LDG2) was recorded or the neurons were directly stimulated with current pulses through intracellular pipettes (5–10 MΩ) coupled to an electrometer (W-P Instruments, Inc.). Gill potentials were sensed with flexible-tip suction electrodes (placed on anterior gill pilnules or the efferent vein of the gill where they do not affect gill mobility), amplified with a Tektronix 122, and then displayed on a Grass polygraph.

Gill withdrawal was monitored, with occasional photographs and routinely with a Grass (model Ft .03) tension transducer. A thread, in series with an elastic strand, was attached to the base of several gill pilnules in the middle of the gill and to the transducer. Gill withdrawal was measured on the polygraph. An amplitude of 50 mm (1 mV) was equal to a force of 0.33 g and the equivalent of a full gill withdrawal (as shown in Fig. 5 of ref. 11) in most preparations. A comparison of the transducer record of gill withdrawal with photographs showed good qualitative agreement.

Muscle junctional potentials were recorded from the longitudinal muscles of the efferent vein of the gill (14) with microwires (>30 MΩ, 2.5 M KCl) in six separate preparations. The efferent vein was slit longitudinally and pinned out so that the muscle cells could be penetrated to record the EJPs while
gill potentials were also recorded and central motor neurons were selectively activated.

RESULTS

In assessing the performance of motor neurons during habituation, we recorded the intracellular activity of L7 and LDG1 and simultaneously made extracellular recordings from the gill. The latter technique, first used by Peretz (16, 17), yields information on the firing of motor neurons and their effect on gill muscles. We noted that the amplitude of the gill potentials changed systematically with the firing of motor neurons during habituation.

EJP Facilitation and Correlated Gill Potentials. The large frequency-dependent changes in amplitude of the gill potentials observed during habituation suggested that they were caused by muscle junctional potentials. To verify this, from the longitudinal muscles of the gill efferent vein we recorded both the EJPs and the gill potentials evoked by selective intracellular current pulses in LDG1 (Fig. 1). LDG1 was hyperpolarized to prevent spontaneous activity for 30 sec prior to the first evoked EJP in Fig. 1. Therefore, the first EJP represents the unfacilitated state. It facilitated to twice the initial amplitude with low frequency (about 1/sec) activity. After a 6-sec break in the record, the amplitude had not completely decayed and higher frequency activity (about 4/sec) caused pronounced facilitation of the EJPs to 14 mV or 3 times the unfacilitated amplitude. Subsequent LDG1 pulses showed that the EJP amplitude decayed slowly. The gill potential amplitude followed the EJPs in a linear manner and is an accurate representation of the EJP amplitude. Our observations on EJPs are in general agreement with earlier studies (14). We observed EJPs with unfacilitated amplitudes from 2 to 7 mV and facilitated amplitudes of up to 20 mV from 45 impalements (six preparations) of effenter vein longitudinal muscle fibers with resting potentials of 60–70 mV. Dual innervation of some fibers (LDG1 and likely LDG2) and small inhibitory junction potentials were also observed. Some fibers were held for 10–20 min so facilitation and its decay could be studied.

We tested the decay of EJP facilitation by evoking activity in LDG1 with 5-sec current pulses at 30-sec intervals. As shown in Fig. 2, spikes in LDG1 at 2/sec for 5 sec produced facilitation of the EJPs to 3.4 times their initial amplitude. The EJPs decayed to half of the fully facilitated amplitude in 6 sec and had decayed completely in 25 sec. If LDG1 was allowed to fire at about 1/sec after the pulse, the facilitation did not decay completely but was maintained in a steady state at 2.5 times the unfacilitated amplitude. Firing at 0.5/sec produced a steady state at twice the unfacilitated amplitude and firing at 2/sec produced a steady state at 3.4-fold. Firing at frequencies higher than 5/sec usually caused sufficient contraction of the muscle fiber to dislodge the electrode from the muscle. The upper limit of facilitation amplitude was estimated to be 4–5 times the unfacilitated EJP from gill potential recordings at high frequency.

Gill Withdrawal Dependence on Facilitation of Gill Potentials. The influence of frequency-dependent facilitation at the gill neuromuscular junctions on gill withdrawal was tested by selective stimulation of LDG1 with intracellular current pulses while simultaneous recordings were made of LDG1, L7, gill potentials, and gill withdrawal. As shown in Fig. 3 (P1), pulses at 8 spikes/sec in LDG1 produced pronounced facilitation of the gill potentials. Gill withdrawal began to rise only after considerable facilitation of the gill potentials. P2, delivered 30 sec later, was preceded by low-frequency activity in LDG1 evoked by releasing LDG1 from a slight hyperpolarization. This antecedent activity served to facilitate the gill potentials somewhat so that the gill potentials in P2 started from a more facilitated state and reached a larger final amplitude. The result was a much greater (4-fold) gill contraction than in P1. At P3, 30 sec later, the pulse of LDG1 spikes had substantially the same effect as P1, showing that the facilitated state in P2 had completely decayed in the 30-sec interval.

The effect of antecedent activity in LDG1 prior to P2 is similar to the effect of a dishabituation stimulus because it promotes firing in motor neurons and the facilitation of EJPs. Thus, the gill withdrawal evoked by a spike train during a habituation depends upon the frequency of the evoked spikes (because of the frequency-dependent facilitation of the gill muscle EJPs) and the facilitated

* Selective current pulses applied to motor neurons at constant intervals (10 or 30 sec) may cause relatively stable gill withdrawal (14). We found that the stability depended upon the firing frequency. High frequencies (17/sec for 2 sec in L7, 14/sec for 3 sec in LDG1) caused gill withdrawal to decrease after repeated trials [Jackett, J. W. & Rine, J. (1975) The Physiologist 18, 260 and Neurosciences Abstr. 1, 585]. Subsequent analysis showed reduced gill potential amplitude during decrease of gill withdrawal at these frequencies, suggesting that EJPs facilitate less after repeated trials of high-frequency activity.
state of the initial evoked EJP, which is determined by antecedent activity.

Contribution of EJP Facilitation to Habituation and Dishabituation. We simultaneously recorded the activity of motor neurons L7 and LDG1 (10 animals with dual impalements, others with single) and the gill potentials during habituation of gill withdrawal evoked by stimulation of the siphon. These neurons were identified by position in the ganglion, characteristic motor effects, and interneuron input (11). It was found that the number of spikes evoked in these neurons on trial 5 of a habituation run was reduced to about 60% of trial 1 when the gill withdrawal had fallen to 30% of trial 1 (Table 1). Also, after dishabituation, the increase in spikes was disproportionately smaller than the increase in gill withdrawal (0.2 increased to 0.9).

The disproportionate decrease in gill withdrawal during habituation and the disproportionate increase in gill withdrawal after dishabituation could be accounted for by frequency-dependent facilitation at the neuromuscular junctions as revealed by gill potential recordings. An example of the frequency-dependent changes in the amplitude of gill potentials (evoked by EJPs) during habituation trials is shown in Fig. 4. In this case the gill electrode selectively recorded the EJPs evoked by LDG1, because a one–one correspondence was observed. The first gill potential (arrow) in T1 was unfacilitated, since LDG1 had not fired for 30 sec prior to T1. Successive gill potentials evoked in T1 facilitated to larger amplitudes dependent upon frequency. On trials 2–7 the withdrawal decreased as well as the number of spikes (as in Table 1). A dishabituation stimulus was given between T7 and T8 which caused extensive firing of the motor neurons (13 LDG1 spikes/2 sec at dishabituation and 6 LDG1 spikes/10 sec just prior to T8), comparable to dishabituation firing observed by others (19). The facilitation of the gill potentials, provoked by this firing, had not decayed at T8 because the first evoked gill potential in T8 was 4 times the amplitude of the first gill potential in T1. Subsequent gill potentials increased in amplitude according to the firing frequency of LDG1. Now, each gill potential having facilitated to a large amplitude gave rise to a large, maintained gill withdrawal with superimposed discrete twitches of gill withdrawal for each LDG1 spike. Fig. 4 shows that the gill potentials behaved during habituation as expected from our previous analysis of selective firing of LDG1 (i.e., Figs. 1 and 3)—that is, high-frequency firing of motor neurons caused

![Fig. 3. Enhancement of gill potentials and gill withdrawal by antecedent activity in neuron LDG1. LDG1 was selectively stimulated at 30-sec intervals (P1, P2, P3) while gill potentials (G-P), gill withdrawal (G-T), and LDG1 and L7 were recorded. L7 was unaffected by firing LDG1, as found by others (11). Note the delayed slow rise of G-T in P1 and P3 and its earlier rapid rise associated with rapid facilitation of G-Ps in P2.](image)

### Table 1. Changes in evoked spikes (2-sec) and relative gill withdrawal during habituation and dishabituation

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th>Trial 5</th>
<th>Trial pre. dis.*</th>
<th>Trial post. dis.†</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDG1</td>
<td>9.5</td>
<td>6.7</td>
<td>5.1</td>
<td>4.5</td>
</tr>
<tr>
<td>L7</td>
<td>10.5</td>
<td>5.8</td>
<td>6.0</td>
<td>5.1</td>
</tr>
<tr>
<td>G-T</td>
<td>1.0</td>
<td></td>
<td>0.5</td>
<td>0.2</td>
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Intertrial interval, 30 sec; 12 observations for each mean.

All animals (eight with dual impalements) gave moderate to large gill withdrawal (G-T) on trial 1. Dishabituation (mantle stimulus) usually, but not always, was interposed between trials 7 and 8.* The trial just prior to dishabituation.† The trial just after dishabituation.

rapid facilitation and consequent summation of EJPs which led to disproportionately large gill withdrawal. The withdrawal (T8 of Fig. 4) was also strongly influenced by the facilitated state of the initial EJP as observed in Fig. 3 (P2) during selective firing of LDG1.

### DISCUSSION

The number of evoked spikes in motor neurons L7 and LDG1 on the average was reduced to half by trial 5 of a habituation run. Our data from recording of LDC2 and gill potentials suggest that other motor neurons are behaving in a similar manner. These findings agree with earlier observations on the firing of L7 (9). Central motor neurons are responsible for most of the gill withdrawal in response to weak-moderate intensity stimulation of the siphon. A peripheral pathway from siphon to gill contributes greatly (15) after effenter nerves from the central nervous system are cut and the appropriate stimulus is used. When the effenter nerves are intact, as in this study, the peripheral pathway contribution is minimal [estimated 14% at 10 g (15) and 5–15% (10)].

Gill potentials are caused by EJPs as shown by the simultaneous recording of these potentials during selective firing of central neuron LDG1. The EJPs evoked by other central motor neurons may be recorded as gill potentials by the appropriate placement of the gill electrode (i.e., pinnules for L7, effenter vein for LDG1). Thus, one may follow the relative changes in amplitude of EJPs evoked by specific motor neurons during behavioral habituation. Facilitation of EJP amplitude occurs at low frequencies (ca. 0.3/sec), is very frequency-dependent, and decays slowly. Low-frequency EJP activity between bursts of EJPs can maintain the facilitated state of the EJPs and thus augment the facilitation of subsequent bursts. All the EJP changes we have studied are explained by homosynaptic facilitation.

The neuromuscular junctions in the gill of *Aplysia* are not

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*Joint experiments at Kandel's laboratory in January 1976 [by Carew, T., Castellucci, V., Byrne, J. & Kandel, E. (1976) *Neurosciences Abst.* 2, 317] confirmed findings (15) that, after removal of the central nervous system, the gill withdrawal mediated by the peripheral pathway is, on the average, as large as the response with the central nervous system intact. Differences in findings between the laboratories were agreed to be 2-fold: (i) a "tapper" stimulator (15) evoked large responses after central nervous system removal, a "probe" stimulator (12) did not, and the WaterPik (8, 10) was not tested; (ii) they selected animals (about 50% of those prepared) that gave large responses. In ref. 15, all healthy responsive animals prepared for experimentation were considered. With the central nervous system intact, both stimulators activate central neurons with minimal contribution of the peripheral pathway at weak-moderate intensity stimuli.*
unique in showing homosynaptic facilitation during habituation. In the crayfish, depression at the motor-giant flexor muscle synapse occurs rapidly at low (1/1 min) stimulus rates (20). It can be reversed or facilitated by increasing the stimulus frequency to 0.1/sec. During behavioral habituation this synapse declines in effectiveness rapidly and leads to flexor reflexes that are attenuated somewhat in strength but the other fast flexor motor neuron-flexor muscle synapses are stable or facilitate so that the total motor system continues to be effective with repeated activation (2, 3).

Gill withdrawal evoked by central neuron activity is very frequency-dependent, beginning only after considerable high-frequency activity of central neurons and the corresponding facilitation of EPJs. Low-frequency activity does not produce gill withdrawal (threshold is 6 spikes/sec in L7 (11) and 4 spikes/sec in LDG1) but individual low-frequency spikes in LDG1 may produce correlated twitches of gill withdrawal after a high-frequency burst of activity facilitates the neuromuscular junctions (i.e., Fig. 4, T8). Individual twitches were also observed by Kupfermann et al. (11) using a photocell recording of gill withdrawal.

The synaptic sites involved in habituation and dishabituation of the gill withdrawal reflex evoked by siphon stimulation are summarized in Fig. 5. Clusters of central sensory neurons (12) convey information about siphon stimulation to central motor neurons (7, 9, 11) via chemical synapses (site 1 in Fig. 5). During habituation these synapses undergo synaptic depression (13, 19) and the number of evoked spikes in motor neurons falls to about half in five trials. On early habituation trials, high-frequency prolonged motor neuron activity causes facilitation of gill neuromuscular junctions (site 2 in Fig. 5), which decays slowly between trials and is slowed more by motor neuron activity between trials. Successive habituation trials evoke fewer neuronal spikes and less activity between trials, which results in less facilitation at neuromuscular synapses and diminished withdrawal. On later trials the frequency of evoked spikes may be below threshold for withdrawal and thus a motor neuron “drops out.” During dishabituation, motor neurons fire extensively (18, 19) and cause facilitation of neuromuscular junctions. The facilitation decays over 10s of seconds or longer, depending on the amount of firing. Habituating trials, after the dishabituation, evoke spike trains in motor neurons which are augmented by homosynaptic facilitation at central neurons (site 3 in Fig. 5; 18, 19). These spike trains act on neuromuscular junctions that are already facilitated by the dishabituating activity and therefore their effect on gill withdrawal is greatly enhanced (i.e., T8 in Fig. 4). At subsequent trials, motor neurons decrease their firing, due to central synaptic depression, which results in less facilitation of neuromuscular junctions and a rapid decline in gill withdrawal. Other phenomena at the gill (site 5 in Fig. 5) may contribute to habituation.

In studies of the potentiation of central synaptic potentials in L7 after dishabituation (7, 19) the EPSPs reach maximal amplitude less than 20 sec after dishabituation. Therefore, the number of spikes in L7 and LDG1 and gill withdrawal should be greatest at that time. We find this to be the case, but the motor neurons change their output only slightly on the average, as observed by others also (9), and L7 may paradoxically decrease its firing occasionally. The large change in gill withdrawal evoked by a habituating stimulus after dishabituation can be accounted for by the elevated facilitation state of neuromuscular junctions caused by the dishabituating stimulus. Any antecedent activity in a central motor neuron, evoked by external stimuli (i.e., dishabituation) or a central program (16, 26), can enhance the gill withdrawal evoked by that neuron because of the frequency-dependent neuromuscular facilitation.

Our studies identify the neuromuscular synapses as sites of plasticity in the gill withdrawal reflex. These synapses facilitate and produce a relatively stable gill withdrawal from trial to trial if they are tested with constant-frequency spike trains (14). However, the important amplifying effect of facilitating synapses becomes apparent when the frequency and duration of spike trains change as they do during habituation and after dishabituation.

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