Sensitization of the Gill and Siphon Withdrawal Reflex of Aplysia: Multiple Sites of Change in the Neuronal Network

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SUMMARY AND CONCLUSIONS

1. Recent studies have emphasized the major contribution of interneuronal transmission to the mediation and learning-associated modulation of the gill and siphon withdrawal (GSW) reflex of Aplysia. We wish to provide more direct support for the hypothesis that inhibitory junctions are crucial sites of plasticity.

2. In parallel experiments we investigated modulation at five major sites of synaptic transmission in the GSW network: 1) from sensory neurons to motor neurons, 2) from sensory neurons to excitatory interneurons (INTs+), 3) from INTs+ to motor neurons (MNs), 4) from inhibitory interneurons (INTs-) to INTs+, and 5) from INTs+ to INTs-.

3. While recording simultaneously from a single sensory neuron of the LE cluster, an INT+, and a MN, we found that both LE-MN and LE-INTs+ synapses were facilitated by the activation of modulator neurons by stimulation of the left pleuroabdominal connective (185% and 93%, respectively) as well as by serotonin (5-HT) (191% and 84%). Junctions of the second type were therefore less facilitated. The difference in the magnitude of facilitation at these two sites is an indication of a branch-specific, differential efficacy in the modulation of different central synapses made by a single neuron.

4. Although INT+ -MN junctions have the capacity to display marked posttetanic potentiation, they are not significantly potentiated after connective stimulation. Sensitization of the GSW reflex is therefore not necessarily accompanied by a modification of transmission at these synapses.

5. Inhibitory transmission to INTs+ is significantly reduced by connective stimulation (36%) and by 5-HT (71%). This supports the hypothesis that a reduction of feedback inhibition onto INTs+ is a major mechanism of reflex sensitization and may account for the increased evoked firing of INTs+ that is observed after connective stimulation.

6. The excitatory input to INTs- is selectively decreased by 5-HT (50%) and by the molluscan neuropeptide small cardioactive peptide B (38%). This latter effect, which could produce disinhibition of INTs-, may explain the previous observation that this peptide is able to potentiate the evoked input to MNs of the reflex at a concentration (1 μM) that fails to modify monosynaptic sensory-motor transmission.

7. These results indicate that transmission through a small neuronal network that mediates a withdrawal reflex in Aplysia may be modulated at multiple sites and by different mechanisms. These mechanisms include: 1) branch specific facilitation of sensory neuron outputs and 2) inhibition of INT -INT+ inhibitory postsynaptic potentials by endogenous modulatory neurons and by 5-HT. Parallel modulation at each of these sites is likely to contribute to sensitization of the GSW reflex.

INTRODUCTION

Transmission within neuronal networks is known to be a highly plastic process. It can be modified according to changes in the behavioral status of an animal. In the lobster, for example, recent studies have shown that transmission through the pyloric network is reorganized after brief sensory input from the stomach (Hooper and Moulins 1990). Learning is also evidently associated with changes in synaptic transmission. Studies of long-term potentiation (LTP) in the hippocampus, a candidate mechanism of memory formation, have, for example, demonstrated that transmission through the perforant path may be increased after high-frequency activation of this afferent pathway to pyramidal cells (Bliss and Lomo 1973). Although most studies on this phenomenon have focused on changes in transmission at the principal excitatory junctions of the circuit (Lynch and Baudry 1991; Lynch et al. 1983; Malinow and Tsien 1990), modifications at other sites such as inhibitory synapses could also play a role (Maru et al. 1989; Misgeld et al. 1979; Mott and Lewis 1991; Mott et al. 1993; Stelzer et al. 1987; but see Griffith et al. 1986). Technical difficulties have, however, prevented an adequate evaluation of the role of parallel modifications at excitatory and inhibitory synapses of the hippocampal circuitry in producing LTP. Such an issue is more easily approached in the nervous system of invertebrates, where most of the elements of a small circuit can be identified, thereby allowing parallel investigations of the plastic properties of the various synapses of the network. An adequate model system is found in the withdrawal reflexes of Aplysia.

The gill and siphon withdrawal (GSW) reflex of Aplysia californica is mediated by both centrally and peripherally located neurons (Byrne et al. 1974; Colebrook and Lukowiak 1988; Dubuc and Castellucci 1991; Kanz et al. 1979; Kupfermann et al. 1974; Lukowiak and Peretz 1977; Zecevic et al. 1989). Attempts to understand learning-associated changes of this behavior have mainly focused on the central component, which is located in the abdominal ganglion. In this ganglion, the neuronal network mediating the GSW reflex comprises a monosynaptic pathway (i.e., sensory-motor synapses) and a polysynaptic pathway. The latter contains both excitatory interneurons (INTs+) and inhibitory interneurons (INTs-), some of which have been previously described (Byrne 1981; Frost et al. 1988; Hawkins et al. 1981a,b). It is known for example, that INTs+ 1.34, 1.29, 1.28, 1.23, and 1.22 are directly excited by sensory
neurons of the LE cluster and in turn synapse on siphon and gill motoneurons. The synaptic organization and functional properties of the polysynaptic pathways, however, remain incompletely defined.

Sensitization of the GSW reflex is a nonassociative form of enhancement of the nonhabituated reflex induced by a noxious stimulus to the tail or the head of the animal. Enhancement of a previously habituated response is referred to as dishabituation. It has been found that transmission at LE-motor neuron (MN) synapses is increased after protocols that lead to both short- and long-term reflex enhancement (Brunelli et al. 1976; Castellucci and Kandel 1976; Castellucci et al. 1970; Montarolo et al. 1986). The molecular mechanisms of this facilitation are now partially understood (Bailey et al. 1992; Dash et al. 1990; Goldsmith and Abrams 1991; Hochner et al. 1986a,b; Klein 1993; Klein et al. 1982; Mayford et al. 1992; Pieroni and Byrne 1992).

To give a satisfactory account of the neurophysiological basis of short- and long-term learning-associated modifications in this simple behavior, one must identify all important synapses that are modified. At present, only the monosynaptic sensory-motor connections between LE sensory neurons and a subset of MNs (L7 and those of the LFS group) have been well studied in this respect.

Attempts to explain sensitization and dishabituation have focused on the polysynaptic component of the neuronal network because it was thought that >60% of the evoked afferent input to the central MNs was directly mediated by sensory-motor connections (Byrne et al. 1978). Our recent work has, however, emphasized the role of the interneurons of the network in the mediation and plastic properties of the GSW reflex. We have found that only ∼25% of the input to MNs evoked by tactile stimulation of the siphon skin or electrical stimulation of the siphon nerve is mediated monosynaptically (Trudeau and Castellucci 1992). Because polysynaptic transmission is so important, it appears plausible that at least some of the synaptic changes associated with sensitization occur at interneuronal synapses. This idea is supported by our finding that the molluscan neuropeptide small cardioactive peptide B (SCPb) can potentiate evoked input to the MNs at a concentration that does not affect sensory-motor connections (Trudeau and Castellucci 1992).

A crucial locus of learning-associated synaptic modification in the polysynaptic component of the GSW reflex network appears to be the INTs+. (Fischer and Carew 1993). Most described inhibitory synaptic transmission in the CNS of Aplysia is mediated through acetylcholine gated Cl- channels and has a well-described pharmacology; the classical nicotinic blocker d-tubocurarine (d-TC) is an effective antagonist at such synapses (Kehoe 1972; Taue and Gerschenfeld 1962). Using d-TC, we have recently provided evidence for the involvement of feedback inhibition on the INTs+ of the network as an important determinant of evoked gill contractions (Trudeau and Castellucci 1993). The INTs+, which are directly excited by the sensory neurons, receive powerful inhibitory input after the initial sensory drive. Application of d-TC blocks this inhibition, thereby considerably increasing the excitability of INTs+. This treatment also prolongs siphon-evoked excitation of the MNs as well as gill contractions. In preparations pharmacologically disinfhibited by pretreatment with d-TC, the facilitation of siphon-evoked input to MNs by pleuroabdominal connective stimulation (a cellular analogue of sensitization) is considerably reduced; this indicates that a major mechanism of sensitization may be the functional uncoupling of the INTs+ that mediate feedback inhibition in the circuit.

We wished to test more directly the hypothesis that a major mechanism of sensitization is a reduction of inhibitory transmission at synapses between INTs− and INTs+ of the neuronal network that mediates the GSW reflex. A previous report has already provided some indication that such a modulation can take place at synapses from L30 INTs− to facilitator neurons L29 (Frost et al. 1988). In the present report, the effects of pleuroabdominal connective stimulation (an analogue of sensitizing input) and of the neuromodulators serotonin (5-HT) and SCPb were investigated at five principal types of synaptic junctions of the GSW reflex circuit (Fig. 1): 1) synapses from sensory neurons to MNs, 2) synapses from sensory neurons to INTs+, 3) synapses from INTs− to INTs+, 4) synapses from INTs− to MNs, 5) the junctions responsible for the excitation of INTs+. In the present report, because the EPSPs evoked by sensory neurons were minimally depressed before application of the treatments, we refer to the synaptic facilitation as contributing to reflex sensitization. Our results demonstrate multiple, parallel sites of change; together with our previous reports, they suggest that sensitization of the GSW reflex is not fully accounted for by an increase in sensory motor transmission, and that a decrease of feedback inhibitory input on INTs+ may play a determinant role in short-term enhancements of the GSW reflex associated with learning.

**METHODS**

**Preparation**

*A. californica* (150–300 g) were purchased from Marinus (Venice, CA) and maintained in large 900-l tanks at 15°C. All experiments were performed at room temperature (22°C) on isolated
abdominal ganglia. Before dissection, animals were anesthetized with an injection of MgCl₂ (385 mM) corresponding to approximately one third of their volume. Dissection of the abdominal ganglion was performed in an extracellular medium made from equal volumes of 385 mM MgCl₂ and artificial seawater (ASW). The ganglion was pinned to the bottom of a Sylgard-coated chamber (2 ml) and the sheath was removed with fine forceps. A long section of both pleuroabdominal connectives, siphon nerve, and branchial nerve was kept intact and aspirated into suction electrodes. Before experiments were begun, all preparations were rested for ≥2 h under constant superfusion of ASW.

**Electrophysiology**

Sharp microelectrodes were pulled from omega-dot borosilicate glass (WPI, Sarasota, FL) and filled either with 3 M KCl or 3 M KAc. Their resistances were between 10 and 20 MΩ. Patch electrodes were pulled in two stages from Boralex glass (Rochester Scientific, Rochester, NY) and were not fire polished. Their internal solution was in mM KCl, 2 MgCl₂, 100 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 10 bis-(o-aminophenoxo)-N,N,N',N'-tetraacetic acid, 5 Na,GTP, 10 SH-glutathione, and 100 glucose, pH 7.3. Resistance before seal formation was 1–3 MΩ. Voltage signals were recorded through Axoclamp 1A and 2A amplifiers (Axon Instruments). Single-electrode voltage clamp was performed with an Axoclamp 2A amplifier in the continuous mode. The area under compound EPSP traces as well as the peak amplitude of EPSPs, inhibitory postsynaptic potentials (IPSPs) and inhibitory postsynaptic currents (IPSCs) was measured with the Spike data analysis software (Hilal Associates). When EPSPs from sensory neurons to MNs included a polysynaptic component due to the firing of an intercalated INT⁺, only the peak amplitude of the first component was measured. Data are presented as means ± SE and were analyzed by performing analyses of variance (ANOVA). Group means were compared with Tukey tests.

**Drugs and solutions**

ASW was composed of (in mM) 460 NaCl, 10 KCl, 3 CaCl₂, 30 MgCl₂, 25 MgSO₄, and 10 HEPES buffer, pH 7.6. The modified ASW (2.1 ASW), which contained twice the normal concentration of Mg²⁺ and 1.25 times the normal concentration of CaCl₂, was composed of (in mM) 368 NaCl, 8 KCl, 13.8 CaCl₂, 3 Na,GTP, 5 Na,ATP. 0.1 Na,Glu 1 P, 10 SH-glutathione, and 100 glucose, pH 7.6. SCP₃ was obtained from Richelle Biotechnologie (Québec) and 5-HT was from Sigma (St. Louis, MO).

**RESULTS**

In the first series of experiments, simultaneous intracellular recordings were made from three types of neurons: an LE sensory neuron, an INT⁺, and an L-FS MN that was current clamped to ~80 mV. The LFS MNs were chosen for these experiments because it is easier to locate INTs⁺ for these cells than for other MNs such as L7. Because our previous results indicated that both types of MNs receive input that is similarly gated by inhibitory interneuronal transmitters and also similarly modulated by 5-HT, by SCP₃, and by facilitator neurons activated by stimulation of the left pleuroabdominal connective (Trudeau and Castellucci 1992, 1993), the results obtained with LFS MNs should be representative of the modulation displayed by the input to MN L7. However, we cannot presently extend our conclusions to other MNs, such as those of the LDG or LBS groups, which could be differently modulated. Previous reports have indeed presented evidence for differential modulation of MNs involved in the siphon and gill components using modulatory protocols different than the one utilized in our study (Hawkins et al. 1990; Wright et al. 1991).

The INTs⁺ were found within a crescent-shaped region rostral to the LE cluster of sensory neurons. Previous reports have differentiated among L34, L29, and L28 interneurons in this region. We found that these groups of cells (2–4 per group) had very similar properties: 1) they received monosynaptic EPSPs from sensory neurons, 2) they monosynaptically excited LFS MNs, 3) they received a mixture of spontaneous EPSPs and IPSPs, and 4) they received monosynaptic IPSPs from INTs⁻. The same results were obtained with all INTs⁺ tested.

After impalement and a 5-min rest, the sensory neuron and the interneuron were alternately stimulated intracellularly through a bridge circuit; EPSPs were recorded in the interneuron (LE-INT⁺ EPSP) and in the MN (LE-MN EPSP and INT⁺-MN EPSP). Three types of synaptic junctions were therefore monitored in parallel. During these experiments, the MN was polarized to ~80 mV and the INT⁺ to ~50 mV by appropiate current injection. The effect of three treatments that lead to enhancement of these synapses were evaluated on these synapses in different preparations: 1) left pleuroabdominal connective stimulation (a analogue of sensitizing input; see Castellucci et al. 1970, Trudeau and Castellucci 1992), 2) bath application of 5 μM 5-HT, a modulator thought to play an important role in sensitization of the GSW reflex (Brunelli et al. 1976; Glanzman et al. 1989), and 3) bath application of 1 μM SCP₃, a neuropeptide present in the CNS of Aplysia (Abrams et al. 1984; Lloyd et al. 1985). The concentration of 5-HT was chosen to be 5 μM on the basis of past studies that showed that the concentration of 5-HT could produce reliable facilitation of sensory-motor EPSPs (Daw et al. 1990; Klein 1993; Mercer et al. 1991). The neuropeptide SCP₃ was used at 1 μM because we previously found that this concentration is able to potently facilitate the evoked input to LFS and L7 MNs (Trudeau and Castellucci 1992). The effects of these treatments on the two types of LE synapses were analyzed by 2x2 ANOVAs with repeated measures. The first factor was the type of synapse (LE-MN vs. LE-INT⁺), the second factor was the treatment (treated vs. control), and the trial number acted as a third factor (repeated measures).

**Synapses between LE sensory neurons and LFS MNs**

The sensory neuron was stimulated above threshold at an interstimulus interval of 1 min. After the second EPSP in the MN, the left connective was stimulated for 5 s (5 V, 3 ms per shock, 8 Hz) and four additional EPSPs were recorded. This level of connective stimulation reliably facilitates the evoked input to MNs of the reflex (Trudeau and Castellucci 1992). In other experiments, connective stimulation was replaced by bath application of 5-H1 (5 μM) or SCP₃ (1 μM). Because this synapse undergoes prominent homosynaptic depression, it was necessary to compare the effects of the treatments with controls obtained by measuring six successive EPSPs without any treatment (n = 5). The results of these experiments are presented in Figs. 2 and 3. As expected from previous results, connective stimula-
fig. 2. Simultaneous recordings of the synaptic junctions between sensory neurons (of the LE cluster) and INTs+ as well as MNs of the GSW reflex. A: representative intracellular recordings performed in the 3 types of neurons. The synapses were sampled with an interstimulus interval of 1 min. One control trace and 2 experimental sets of traces are shown. Activation of modulator neurons by stimulation of the left pleuroabdominal connective induced a facilitation of excitatory postsynaptic potential (EPSP) amplitude at both synapses. This effect was larger at the sensory-motor synapses. Calibration: 4, 4, and 15 mV, 30 ms. B: similar recordings from a preparation where activation of modulator neurons was replaced by bath application of serotonin (5-HT) (5 PM). The facilitation of EPSP amplitude by 5-HT was also more pronounced at the sensory-motor connections. Calibration: 5, 5, and 15 mV, 40 ms. C: unlike 5-HT, bath application of 1 PM small cardioactive peptide B (SCPb) failed to modify EPSP amplitude at both synapses. Calibration: 8, 8, and 10 mV, 50 ms.

fig. 3. Summary of the results presented in Fig. 2A. Effect of the activation of modulator neurons by stimulation of the left connective on monosynaptic connections between sensory neurons and MNs (LE-MN) as well as between sensory neurons and INTs+ (LE-INT+). Interstimulus interval was 1 min. The treatment was effected after the 2nd EPSP (t). The results (n = 7) were compared with control curves obtained in other preparations without any treatment (n = 5). EPSPs are expressed relative to the initial control of each experiment. Note that the facilitation was larger at the LE-MN synapses.

A

B

C

Synapses between LE sensory neurons and INTs+

It has been previously assumed that synapses from sensory neurons to interneurons would be facilitated by 5-HT or modulator neurons in the same manner as the synapses to the MNs. Because no data have previously been reported on this issue, it was of interest to compare, in parallel experiments, the plasticity of these connections with that of LE-MN synapses. These data were obtained in the same experiments as those described above. As is apparent in Fig. 3, A and B, although both connective stimulation and 5-HT produced significant facilitation, the effects were considerably smaller than at LE-LFS synapses. The ANOVA indicated that there was a triple interaction between the treatment effect, the type of synapse, and the trial number [F(5,100) = 2.4, P < 0.05]: the treatment- and trial-dependent facilitation was therefore significantly different for the two synapses. The four EPSPs after connective stimulation (n = 7) were significantly different from their respective...
untreated controls ($P < 0.01, 0.01, 0.05,$ and 0.01) (Figs. 2A and 3A). The mean increase for the four EPSPs was $93.4 \pm 7.7\%$ above control. This represents $50.5\%$ of the facilitation obtained in parallel experiments at LE-LFS junctions. The first LE-INT+ EPSP after connective stimulation was significantly different from the first LE-MN EPSP after connective stimulation ($P < 0.01$). The LE-INT+ EPSPs were also facilitated by bath application of 5-HT ($n = 7$); the second EPSP was significantly different from its respective control ($P < 0.05$) (Figs. 2B and 3B). The mean increase for the four EPSPs was $83.8 \pm 14.8\%$ above control. This represents $43.8\%$ of the facilitation obtained at LE-MN junctions. The ANOVA on the 5-HT data showed that the triple interaction between the treatment effect, the type of synapse, and the trial number did not reach statistical significance ($F(5,30) = 2.13, P = 0.055$). As for the sensory-motor synapses, the neuropeptide SCP$_n$ (1 $\mu$M) produced no significant facilitation of LE-INT+ connections ($n = 6$) (Figs. 2C and 3C).

**Synapses between INTs+ and LFS MNs**

In the experiments described above, the INT+ was also fired every minute, immediately after stimulating the sensory neuron. Again, the effects of the three treatments on INT+-MN synapses were examined after two control EPSPs. Unlike synapses made by the sensory neurons, these connections are stable and do not undergo significant homosynaptic depression. It was therefore not necessary to compare the EPSP amplitude curves with a control curve. These data were analyzed by a one-way repeated-measures ANOVA. In marked contrast to the synapses made by the sensory neurons (Figs. 2 and 3), we found that left pleuroabdominal connective stimulation caused no significant facilitation of these interneuronal synapses ($n = 7$) (Fig. 4).

Bath application of 5 $\mu$M 5-HT ($n = 7$) produced a marked decrease of EPSP amplitudes. There was a significant overall effect of the treatment ($F(5,30) = 21.3, P < 0.01$). The four EPSPs after exposure to 5-HT were significantly different from the last control EPSP ($P < 0.01$). The average decrease of the four EPSPs was $49.8 \pm 2.0\%$ below control. In all these experiments an additional effect of 5-HT was a slow and long-lasting hyperpolarization (2-6 mV) of the INT+. The resulting decrease in excitability required an increase in the depolarizing current pulses used to trigger an action potential in these neurons. Figure 6A provides an example of an experiment where the current pulse was not adjusted. Bath application of SCP$_n$ (1 $\mu$M) also produced a modest decrease of EPSP amplitude ($n = 6$) (Fig. 4). The effect, however, did not reach statistical significance. The average decrease of the four EPSPs was $15.8 \pm 8.9\%$ below control. At the end of some of these experiments ($n = 7$) the ability of INT+-MN synapses to be enhanced was verified by subjecting the interneuron to tetanic stimulation (3 trains of 10 stimuli at 10 Hz, separated by 1 s). As shown in Fig. 4, a large posttetanic potentiation (PTP) was obtained in all experiments. There was a significant overall effect ($F(5,30) = 15.3, P < 0.01$). All four EPSPs after tetanic stimulation were significantly different from the last control ($P < 0.01$). The mean increase was $89.5 \pm 13.2\%$ above control.

**Synapses between INTs− and INTs+**

In a separate series of experiments, INTs− and INTs+ were simultaneously impaled. The former were stimulated every minute and the effects of connective stimulation, 5-HT, and SCP$_n$ were assessed on inhibitory responses recorded in various INTs−. The INTs− were identified by two electrophysiological criteria: 1) they elicited a monosynaptic IPSP in an INT+ and 2) they were excited by stimulation of the siphon nerve. The second criterion was necessary to ensure that the INT− could function within an inhibitory feedback loop on the INTs+ of the reflex.

This last requirement was crucial because we have repeatedly encountered a large (100 $\mu$m) INT−, situated just beside the L34 INTs+ (in the left rostral quadrant of the ventral side of the ganglion), which produced a powerful IPSP onto INTs−, but which was itself strongly inhibited on siphon cervc stimulation. This neuron has a faint orange rim, receives prominent spontaneous IPSPs, and does not receive antidromic spikes from stimulation of the siphon, genital, and branchial nerves or from the pleuroabdominal connectives. Except for its size and location, this neuron appears to have properties similar to neuron L35 described previously by Frost (1987). Because the existence of this neuron has not been previously reported, we designate it neuron L41.

Most of the relevant INTs− (pale and ~30 $\mu$m diam) that were found were situated close to the rLE cluster of sensory neurons in the rostral region of the left hemiganglion (ven-
We found that pleuroabdominal connective stimulation (either IPSPs or IPSCs) (Fig. 5). The one-way re-

duce of peak IPSP or IPSC (n = 6). Bath application of 5 μM 5-HT also

duced a similar decrement that was, however, longer-lasting (n = 5).

Application of SCPB (1 μM) failed to significantly modify the inhibitory

responses (n = 7). Asterisks: significantly different from the last control

(trial -1). Single asterisk: P < 0.05. Double asterisk: P < 0.01.

The effects of bath-applied 5-HT (5 μM) were qualita-

tively similar to those of connective stimulation. Figure 5 shows that 5 HT (n = 5) produced a considerable decrease in transmission at inhibitory synapses. The overall effect of the treatment was significant [F(5,25) = 13.7, P < 0.01]. The four responses after the application of 5-HT were significantly different from the last control (P < 0.01). The mean decrease was 71.3 ± 3.2% below control. As for connective stimulation, 5-HT produced a decrease in the excitability of INT+. This was clearly associated with a long-lasting slow hyperpolarization of 3–7 mV and resulted in the blockade of action potential firing unless the depolarizing current pulse was increased. In one of the six experiments, the pulse was not increased and the duration of the effect was exam-

ined. We found that the decreased excitability was main-

tral surface) (Dubuc and Castellucci 1991). This region is

known to contain the L30 group of INTs−. In some of these experiments the INT+ was impaled by a sharp microelec-

trode filled with 3 M KAc (so as not to alter the reversal potential of IPSPs) and current clamped at its normal rest-

ing potential (50–55 mV): in these experiments, the IPSP itself was therefore measured. In other experiments, the electrode was filled with 3 M KCl and the reversal potential of IPSPs stabilized at potentials more positive (-20 to -30 mV) than physiological (-60 mV): the interneuron was polarized to -80 mV so that the Cl−-mediated inhibitory response was reversed and appeared as an EPSP. Finally, in other experiments, the INT+ was voltage clamped at -80 mV in the whole-cell mode with a single electrode (see METHODS); the electrode solution contained Cl− so that the IPSP that was recorded reversed between -20 and -30 mV within a few minutes after rupture of the membrane. Because the results that were obtained with these different methods were the same, the data were pooled. Unlike the synapses made by sensory neurons, the inhibitory responses did not undergo significant homosynaptic depression; it was therefore not necessary to compare the results with a control curve. The interstimulus interval was 1 s. We found that at this frequency (1 Hz) the IPSP/IPSC was larger and more stable in peak amplitude than with an interval of 1 min. Four traces were sampled and averaged every minute.

We found that pleuroabdominal connective stimulation (n = 6) induced a clear decrement of inhibitory transmission (either IPSPs or IPSCs) (Fig. 5). The one-way re-

peated measures ANOVA showed that there was a signifi-
cant overall effect of the treatment [F(5,25) = 14.3, P < 0.01]. The four responses after connective stimulation were significantly different from the last control (P < 0.01, 0.05, and 0.05). The mean decrease over the four responses was 36.1 ± 4.4% below control. An additional effect of connective stimulation in most preparations was a decrease in the excitability of the INT−. This was apparent as a blockade of the initiation of action potentials by the depolarizing current pulse; in six experiments the amplitude of the pulse was increased to maintain action potential firing and to be able to measure the postsynaptic response in the INT−. In two other experiments, the pulse was not modified to assess the duration of this effect. Figure 6B shows traces from one of these experiments. This decreased excitability lasted ∼90 s and was not associated with a marked hyperpolarization of the neuron. Its ionic basis was not further investigated in these experiments.

The changes in interneuronal excitability. A: additional effect of 5-HT on transmission at synapses between INTs− and MNs was a slow hyperpolarization of the INT+ by 2–6 mV. This was associated with a reduced excitability of the neuron so that depolarizing current pulses became insufficient to bring the neuron past threshold. Representative traces from 1 experiment are presented. The hyperpolarization itself is not repre-

dented. Calibration: 10 and 10 mV, 60 ms. B: after stimulation of the left connective, INTs− often displayed reduced excitability; this reduction was not accompanied by a hyperpolarization. Representative traces from 1 experiment are presented. Calibration: 60 nS, 20 mV, 50 ms.
tained unless extensive washing out of the 5-HT present in the bath was performed (>20 min).

The effect of 1 μM SCPa was finally evaluated at inhibitory synapses (n = 7). As shown in Fig. 5, the neuropeptide did not produce any significant change in the amplitude of IPSPs/IPSCs.

**Afferent input to INTs**

The last type of synaptic connection to be evaluated was that responsible for the excitatory input to INTs+ after siphon stimulation. In the diagram of Fig. 1, this is represented by INTs+, which are thought to produce EPSPs onto the INTs- after having themselves been activated by the sensory neurons. Although we have simultaneously impaled a fair number of INTs+ and INTs-, we have been unable so far to find strong chemical connections from INTs+ to INTs-. On two occasions an INT+ - INT- EPSP <1 mV was recorded. This suggests the existence of additional, presently unidentified, INTs+. The presence of direct LE-INT- EPSPs was also verified on a few occasions: no such connections were found. This is unlike what has been reported previously for INT- L16 (Hawkins et al. 1981a).

We have attempted to circumscribe the possible location of unidentified INTs+ and INTs- in the ganglion by injecting Lucifer yellow (Stewart 1978) in the known interneurons so as to identify their main projection areas. We found that the projections of INTs+ were predominantly toward the caudal region of the ventral hemimandib lung (close to L1S MNS), and in the opposite direction, to the rostral region (close to L30 INTs+). If other INTs- exist, these may in all likelihood be situated also in the rostral region, perhaps on the dorsal surface of the ganglion. Lucifer yellow injections in the identified INTs+ revealed that these cells send multiple processes toward the middle of the rostral left hemimandib lung (close to L34, L29, and L28 INTs+), as well as in the opposite direction, through the bag cell cluster, in the left pleuroabdominal connective. If other INTs+ exist, these may also be possibly located on the dorsal surface of the ganglion. The finding of a process in the left connective is different from the report of Hawkins et al. (1981a): these authors reported that L30 INTs- have no antidromic action potentials on left connective stimulation.

We have been unsuccessful so far in identifying the INTs+ responsible for activating the INTs-. To be able, nonetheless, to evaluate the plastic properties of the afferent input to the INTs+, we have obtained recordings from INTs- polarized at −80 mV; compound EPSPs were then evoked in these neurons by brief electrical stimulation of the siphon nerve. It appears that these EPSPs were mainly produced through the activation of INTs+ and not directly by sensory neurons; these EPSPs (results not shown) were almost totally blocked by superfusion of an extracellular medium that contained a high concentration of divalent cations (2:1 ASW, see METHODS): we have verified that this medium prevents action potential firing in interneurons but does not decrease monosynaptic transmission (Trudeau and Castellucci 1992). The intensity of siphon nerve stimulation was adjusted so as to produce a 20- to 40-mV compound EPSP in an MN recorded in parallel and polarized to −80 mV to prevent firing (results not shown).

We have previously shown that this intensity produces gill contractions that are ~30% of maximum (Trudeau and Castellucci 1993).

The compound EPSPs were evoked every minute and were stable during the control period. The EPSPs were quantified by measuring the area under the trace. After two to four controls, the left pleuroabdominal connective was stimulated as above, or 5-HT or SCPb was bath applied. Figure 7A shows that connective stimulation (n = 5) produced an increase in the magnitude of compound EPSPs recorded in INTs-. The overall effect, however, did not reach statistical significance. The mean increase for the four EPSPs was $27.5 \pm 4.9\%$ above control. Bath application of 5-HT (5 μM) or of the neuropeptide SCPb (1 μM) produced the opposite effect (Fig. 7, B and C): both caused a marked decrease in the compound EPSPs. Calibration: 10 mV, 100 ms.

**FIG. 7.** Modulation of the input to INTs−. Compound postsynaptic potentials were recorded in INTs- (polarized to −80 mV) on stimulation of the siphon nerve. Interstimulus interval was 1 min. One control (left) and 2 experimental traces (right) are presented. A: activation of modulator neurons by stimulation of the left connective produced a small increase in the size of the postsynaptic potentials (PSPs). Calibration: 7 mV, 100 ms. B: bath application of 5 μM 5-HT induced a clear decrease of compound EPSPs. Calibration: 10 mV, 100 ms. C: bath application of SCPb (1 μM) also caused a marked decrease in the size of compound EPSPs. Calibration: 10 mV, 100 ms.
The experiments described in this study were designed to provide more direct evidence for the involvement of plasticity at the interneuronal level in the synaptic facilitation that is responsible for sensitization of the GSW reflex. We have not performed any behavioral measurements in these experiments. We posit that modifications of the afferent input to MNs should provide an index of the behavioral output, although this is certainly a simplifying assumption. Using a semiintact preparation we have previously shown that an increased afferent input to LFS and L7 neurons, produced by pharmacological disinhibition, was accompanied by a significant facilitation of the input to the MNs. It is possible, for example, that at some intensities of connective stimulation the results of PTP that was caused by the connective stimulation driving the interneurons to a high-frequency discharge. In that study, however, it was not clear whether the intensity of connective stimulation was sufficient to produce facilitation of transmitter release at the terminals of sensory neurons onto MNs (Clark and Kandel 1984). These results and our data suggest that the various terminals of any given neuron may be modulated to different extents as a function of their site of projection. The fact that the effects of 5-HT were similarly branch specific suggests that it is not the distribution of modulator neuron projections that determines the level of modulation, but rather the number or density of presynaptic receptors for the various neuromodulatory substances at the different terminals of a given neuron; it is also possible that the former influences the latter. Another, simpler, possibility that we have not yet examined is that the smaller increase in EPSP amplitude recorded in the INT+ may be the result of a postsynaptic alteration involving a change in input resistance of the neuron.

Role of PTP in reflex enhancement

Previous reports have demonstrated the capacity of synapses between INTs+ and MNs (INT+-MN synapses) to undergo PTP (Frost et al. 1988; Hawkins et al. 1981b). The possibility that these junctions are driven to produce PTP during sensitization, thereby contributing to the increased behavioral output, has been considered. This hypothesis received partial support from a study that reported that synapses between the modulator/excitatory neuron L29 and LFS MNs were facilitated after connective stimulation (Frost et al. 1988). It was also shown that this facilitation was the result of PTP that was caused by the connective stimulation driving the interneuron to a high-frequency discharge. In that study, however, it was not clear whether the intensity of connective stimulation was sufficient to produce significant facilitation of the input to the MNs. It is possible, for example, that at some intensities of connective stimulation some INT+ are driven to produce PTP even though most others are not, and that synapses made by sensory neurons are not facilitated. What was required was an evaluation of the plastic properties of these connections under conditions where 1) the connective stimulation intensity is known to facilitate the evoked input to MNs (Trudeau and Castellucci 1992), 2) the synapses made by sensory neurons are...
facilitated (Figs. 2 and 3A), and 3) a variety of INTs+ are studied (L28, L29, and L34).

Our results, presented in Fig. 4, were obtained under these conditions. They show that although these junctions are able to undergo robust PTP, they are not facilitated by activating modulator neurons through connective stimulation. We found that connective stimulation did not drive the INTs+ at a level sufficient for the generation of PTP because inhibitory input is rapidly recruited and silences these neurons (results not shown). This observation is supported by our finding that INTs- send a process into the left pleuroabdominal connective and are activated by connective stimulation. It is possible that in the study of Frost et al. (1988) the intensity of connective stimulation used did not recruit the INTs-. It is still possible that under some conditions PTP may develop at these junctions and contribute to reflex enhancement. But our results show that facilitation of the input to the MNs can occur without it.

In our experiments we used stimulation of the connectives as an analogue of sensitizing input instead of more natural forms of stimulation to the head or tail of the animal because it produces robust facilitation of the input to MNs with low between-experiment variability. It is true, however, that nerve stimulation may recruit fibers that are not naturally activated or simultaneously activate fibers that are usually activated by different sites of peripheral stimulation. Facilitator neurons such as L29 have been shown to be excited by tail stimulation but inhibited by head stimulation (Hawkins and Schacher 1989), whereas L28 is excited by both sites. Stimulation of the connectives would activate both; it could thus have the advantage of recruiting a larger number of modulator neurons. It may be worthwhile to repeat these experiments using facilitatory protocols such as tactile stimulation of the tail or head of the animal. The plastic properties of INTs+-MN synapses could be different under these conditions.

Application of exogenous 5-HT and SCP, not only failed to facilitate INT+-MN junctions but actually induced a significant decrease in EPSP amplitude (Fig. 4). Another interesting observation was that exogenously applied 5-HT (5 \( \mu \)M) produced a hyperpolarization of INTs+ that resulted in a marked decrease of the excitability of these cells (Fig. 6A). This may explain the recent observation by Carew and colleagues and by ourselves that 5-HT, although facilitating sensory-motor synapses, was unable to facilitate INT+-MN junctions but actually induced a significant decrease in EPSP amplitude--lasting 90 s after the connective stimulation. This effect is also simulated by exogenous 5-HT, although the effect of 3-HT is more persistent. The ionic basis of this phenomenon, which could be a reflection of the same mechanism responsible for the putative decrease in transmitter release at this synapse, is of great interest but has yet to be investigated.

Another mechanism that could be involved in the reduction of feedback inhibition during sensitization is a selective reduction of the excitatory input to INTs+. Although our data indicate that INTs+-MN synapses are not modified by connective stimulation, it is still possible that branch-specific modulation occurs at INTs+-INTs- junctions. Although we have been unable to identify the INTs+ responsible for activating the INTs-, we have evaluated the modulation of the afferent input to INTs- by stimulating the siphon nerve. Our data (Figs. 7 and 8) show that the evoked input to INTs- is not decreased by connective stimulation. This argues against the hypothesis of a decrease of INTs+-INTs- EPSPs during sensitization; the small increase that is actually observed may be explained as resulting from the increased evoked firing of INTs+ that we have previously shown to be caused by connective stimulation (Trudeau and Castellucci 1993).

Role of feedback inhibition in reflex enhancement

Feedback inhibition onto INTs+ has recently been shown to be a crucial determinant of the magnitude of siphon-evoked gill contractions (Trudeau and Castellucci 1993). Pharmacological blockade of the output of presumably cholinergic INTs- of the GSW reflex leads to a considerable potentiation of evoked gill contractions and of compound EPSPs evoked in gill (L7) and siphon (LFS) MNs by siphon nerve stimulation. The reduced inhibition allows the INT+ to respond more vigorously to the sensory input. In our previous report, we demonstrated that in neuronal networks previously disinhibited pharmacologically, facilitation of the evoked input to MNs by connective stimulation is significantly reduced. This suggests that a reduction of feedback inhibition onto INTs+ is an important mechanism of GSW reflex sensitization. From these results, it could be predicted that transmission at inhibitory synapses onto various INTs+ (INTs+-INTs- synapses) will be decreased after the activation of sensitizing input. A previous report showed that IPSPs evoked between interneurons L30 and L29 are reduced by connective stimulation and 5-HT (Frost et al. 1988), but no other data were available on the plastic properties of connections between INTs- and INTs+ of the GSW reflex.

The results presented in Fig. 5 illustrate our finding that connective stimulation, at a level sufficient to induce facilitation of the evoked input to MNs, caused a significant reduction of IPSPs and IPSCs at all inhibitory junctions that were tested. It may be noted that the time course of this effect nicely parallels that of short-term LE-MN EPSP facilitation (Fig. 3). Bath-applied 5-HT also caused a similar effect with, however, a more prolonged time course. These data, together with our previous results, provide strong evidence in favor of the hypothesis that sensitization of the GSW reflex is due to a large extent to a reduction of a feedback inhibition on INTs+. This may result from a reduction of transmitter release at these inhibitory synapses or from a reduction of postsynaptic responsiveness to the transmitter. Because we have found that INTs- send a process into the left pleuroabdominal connective and are directly activated by connective stimulation, the respective contributions of homosynaptic and heterosynaptic modulation in the effect of connective stimulation remain unclear.

An additional and powerful mechanism to reduce inhibitory transmission during sensitization may be to decrease the excitability of INTs- so that they emit fewer action potentials on activation by INTs+. The data presented in Fig. 6B show that this phenomenon actually does occur and lasts ~90 s after the connective stimulation. This effect is also simulated by exogenous 5-HT, although the effect of 3-HT is more persistent. The ionic basis of this phenomenon, which could be a reflection of the same mechanism responsible for the putative decrease in transmitter release at this synapse, is of great interest but has yet to be investigated.
Application of exogenous SCPα, however, caused a marked reduction of compound EPSPs evoked in INTs⁻ by siphon nerve stimulation (Figs. 7 and 8). At low concentrations (1 μM), it therefore appears that these junctions are the preferential site of action of this neuropeptide, because we found SCPα to have almost no effect at the other synapses of the GSW reflex network. These observations may explain our previous finding that SCPα is able to potentiate the evoked input to MNs of the GSW reflex at a concentration (1 μM) that fails to potentiate LE-MN synapses (Trudeau and Castellucci 1992). At higher concentrations, it is known that SCPα is able to cause facilitation of LE-MN connections (Abrams et al. 1984; Trudeau and Castellucci 1992). The data presented in Figs. 7 and 8 also indicate that 5-HT (5 μM) also caused a decrease in the evoked input to the INTs⁻. This observation fits well with the finding that 5-HT is able to decrease transmission at INT⁺-MN synapses (Fig. 4); it appears that 5-HT acts in a similar fashion at INT⁺-INT⁻ synapses. It may also be noted that the hyperpolarization of INTs⁺ caused by bath application of 5-HT (5 μM) (Fig. 6A) may be itself be responsible for the decreased evoked input to INTs⁻.

On the basis of previous works and the results of this paper, we propose that short-term learning-associated enhancement of the GSW reflex, which occurs in sensitization, may be attributable to a large extent to parallel modifi-
cations at four loci within the GSW reflex neuronal network. These are 1) an increase in excitatory transmission at INT⁻-MN synapses, 2) a smaller increase in excitatory transmis-
sion at LE-INT⁺ synapses, 3) a decrease in inhibitory transmission at INT⁻-INT⁺ junctions and a decreased excit-
ability of INT⁺, and 4) an increase in the basal firing rate of MNs as well as PTP at the neuromuscular junctions (Frost et al. 1988; Jacklet and Rine 1977). Although the mecha-
nism(s) responsible for the changes at the first two sites is presently partially understood (Braha et al. 1990; Castellucci et al. 1980, 1982; Goldsmith and Abrams 1991; Hochner et al. 1986a,b; Klein 1993; Klein et al. 1982), the ionic and molecular basis of the modifications that occur at the third and fourth sites remain to be explored.

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