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Intrinsic Neuromodulation in the *Tritonia* Swim CPG: The Serotonergic Dorsal Swim Interneurons Act Presynaptically to Enhance Transmitter Release from Interneuron C2

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Heterosynaptic enhancement of transmitter release is potentially very important for neuronal computation, yet, to our knowledge, no prior study has shown that stimulation of one neuron directly enhances release from an interneuron. Here, we demonstrate that in the marine mollusk *Tritonia diomedea*, the serotonergic dorsal swim interneurons (DSIs) heterosynaptically increase the amount of transmitter released from another interneuron, C2. Stimulation of a single DSI at physiological firing frequencies increases the size of synaptic potentials evoked by C2. This increase in synaptic efficacy is correlated with an increase in homosynaptic paired-pulse facilitation by C2. Thus, it is likely to be due to an enhancement of transmitter release from C2, rather than a postsynaptic action on the followers of C2. This is further supported by the fact that DSI stimulation enhances the strengths of all chemical synapses made by C2 within the swim network, regardless of their sign. Furthermore, DSI enhances the amplitude of C2 synaptic potentials recorded in neurons that DSI itself does not synapse with. Finally, DSI differentially modulates different synaptic inputs to the same postsynaptic target; while increasing C2-evoked EPSPs it simultaneously decreases the size of EPSPs evoked by other DSIs. The heterosynaptic facilitation of C2 synaptic potentials by DSI is not caused by a simple depolarization of C2, but may be a direct action on the transmitter release mechanism. This neuromodulatory effect, which is intrinsic to the circuitry of the central pattern generator for escape swimming in *Tritonia*, may be important for self-reconfiguration of the swim motor network.

[Key words: 5-HT, heterosynaptic facilitation, nudibranch mollusk, presynaptic mechanism, neuromodulation, central pattern generator]

Heterosynaptic regulation of neurotransmitter release allows a level of fine control for neuronal circuits. For example, reduction of transmitter release from primary sensory neurons by presynaptic inhibition is a nearly ubiquitous mechanism for gating afferent input to central circuits (Sillar, 1991; Watson, 1992; Nusbaum, 1994). Similarly, enhancement of transmitter release from sensory neurons via heterosynaptic facilitation is involved in sensitization of reflexes in mollusks (Hawkins et al., 1981; Mackey et al., 1989; Byrne et al., 1991). Presynaptic enhancement of release is potentially an important mechanism of plasticity in *interneuronal* circuits as well. Yet, surprisingly we have been unable to find examples where neuromodulatory neurons have been directly shown to enhance transmitter release from interneurons. There are many cases where application of exogenous neuromodulatory substances have been shown to cause an increase in neurotransmitter release from neurons (Langer, 1980; Kravitz et al., 1981; Chesselet, 1984; Starke et al., 1989; Delaney et al., 1991; Mintz and Korn, 1991; Cameron and Williams, 1993; Gereau and Conn, 1994; Katz et al., 1994). Although it would appear likely that the neuronal sources of these substances would have similar effects, the evidence that neuronal activity can heterosynaptically enhance release from interneurons is, at present, still indirect (Shimahara and Tauc, 1976; Mintz et al., 1989; Mintz and Korn, 1991). Here we demonstrate that serotonergic interneurons intrinsic to a central pattern generator (CPG) circuit in the mollusk, *Tritonia diomedea*, enhance transmitter release from another CPG interneuron.

Most examples of neuromodulation in motor systems involve sources of neuromodulatory input extrinsic to the local circuit (Harris-Warrick, 1988; Katz and Harris-Warrick, 1990; Harris-Warrick and Marder, 1991; Jordan et al., 1992; Orchard et al., 1993). However, in some systems neuromodulation is intrinsic to the local circuit itself (Cropper et al., 1987; Willows et al., 1988; Nusbaum et al., 1992; Barnes et al., 1994; Katz et al., 1994; Marder, 1994). When the modulatory neurons are extrinsic to the local circuit, the circuit can operate in modulated or unmodulated modes. Furthermore, different extrinsic sources of neuromodulation can affect the circuit at different times, reflecting activity states in other parts of the nervous system. In contrast, when neuromodulation is intrinsic to the circuit it potentially is always present, and the level of modulation is intimately linked to the level of neuronal activity within the circuit itself.

Neuromodulation is intrinsic to the CPG that controls escape swimming in the nudibranch mollusk, *Tritonia*. *Tritonia* is a bottom-dwelling marine sea slug that produces an escape swim in response to contact by a predatory starfish (Willows and Hoyle, 1969; Willows et al., 1973a; Getting, 1983a; Getting and Dekin, 1985a). The escape swim, consisting of a prolonged series of ventral and dorsal whole-body flexions, is generated by a CPG circuit formed by premotor interneurons (Taghert and

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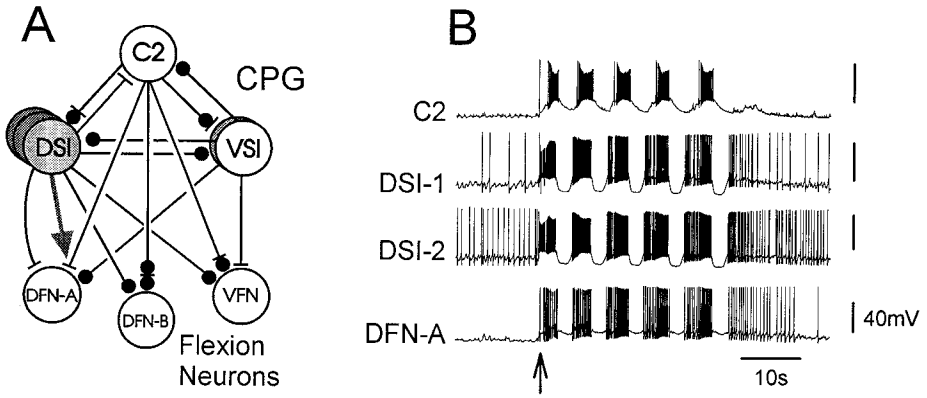

Figure 1. The *Tritonia* swim network and swim motor program. *A,* A simplified wiring diagram showing the important connections among the central pattern generator (*CPG*) neurons, the dorsal swim interneurons (*DSI*), ventral swim interneurons (*VSI*), and cerebral neuron 2 (*C2*) and their connections to the efferent flexion neurons: the dorsal flexion neurons (*DFN-A, DFN-B*) and the ventral flexion neurons (*VFN*) (Getting et al., 1980; Getting, 1981, 1983b; Hume and Getting, 1982). There are three DSIs and two VSIs and a single C2 on each side of the brain. DSI modulates the strength of the connection from C2 to DFN-A (denoted by *arrow*). *Bars* indicate excitatory synapses; *circles* are inhibitory synapses. Multicomponent synapses are shown as combinations of *bars* and *circles. B,* An example of the swim motor program generated by stimulation of pedal nerve 3 (10 Hz, 1 sec at *arrow*). Simultaneous intracellular recordings show that C2, two DSIs, and a DFN-A exhibited five cycles of action potential bursts, which would underlie five cycles of swim activity in the intact animal.

Willows, 1978; Getting et al., 1980; Getting, 1981, 1983b, 1989a) (Fig. 1). These interneurons, the dorsal swim interneurons (DSI-A,B,C), the ventral swim interneurons (VSI-A,B), and peptidergic cerebral neuron 2 (C2), synapse with efferent flexion neurons (Hume and Getting, 1982; Hume et al., 1982) (Fig. 1*A*). Recently, the DSIs were shown to be 5-HT immunoreactive and to have neuromodulatory effects in addition to their classical synaptic actions (Katz et al., 1994; McClellan et al., 1994); stimulation of a single DSI at physiological firing frequencies enhances the amplitude of the synaptic potentials evoked by C2 onto other DSIs and onto DFN-A (Katz et al., 1994). This neuromodulatory effect is due to the actions of 5-HT released from DSI (Katz and Frost, 1993). Determining whether DSI acts presynaptically on C2 to enhance release of its neurotransmitter, or postsynaptically on the targets of C2 to enhance their responsiveness to C2, has important implications for the organization and function of the modulation in the swim network.

Portions of this work have been previously presented in abstract form (Katz and Frost, 1994).

Table 1. List of C2 and DSI synaptic connections in the swim network and the effect of DSI stimulation on C2-evoked PSPs

Post-synaptic neuron	C2 synaptic action	DSI synaptic action	DSI enhancement of C2-evoked PSP?
DSI-A	I	E	Yes (n = 2)
DSI-B,C	E-I	E	Yes (n = 11)[a]
VSI-A	I-E	E-I-E	Yes (n = 3)
VSI-B	E-I	I	Yes (n = 2)
DFN-A	E	E	Yes (n = 48)[a]
DFN-B	I-E-I	I-I	Yes (n = 3)
VFN	E-I	I	Yes (n = 4)

I, Inhibitory; E, excitatory; multicomponent synapses are displayed as combinations of E and I.

[a] Includes some data from Katz et al., 1994.

Materials and Methods

Tritonia diomedea were obtained from the intracoastal waters of Washington State and British Columbia. The central ganglia, consisting of the fused cerebro-pleural and pedal ganglia, were dissected from the animal as described elsewhere (Willows et al., 1973b) and pinned in a Sylgard-lined chamber containing normal saline chilled to 2°C. Normal saline consisted of (in mM): 420 NaCl, 10 KCl, 10 $CaCl_2$ 50 $MgCl_2$, 10 HEPES (pH 7.6), and 11 D-glucose. Throughout the experiment the ganglia were continuously superfused with cooled saline.

To facilitate multiple microelectrode penetrations, the ganglionic connective tissue sheath was removed using fine forceps and scissors. Suction electrodes were placed on left and right pedal nerves 3 (Willows et al., 1973b) for use in nerve stimulation to evoke swim motor programs. A suction electrode on the pedal-pedal commisure (pedal nerve 6) was used to monitor C2 spike activity (Longley and Longley, 1987). Following the dissection and placement of suction electrodes, the preparation was warmed to 10°C and left to rest for at least 3 hr to allow any long-term physiological effects of the dissection procedure to dissipate. All experiments were conducted at 10°C.

Neurons were impaled with glass microelectrodes (8–20 MΩ) filled with 4 M K acetate and inserted into electrode chucks filled with 3 M KCl. Neurons were identified based on soma location and coloration, activity at rest and during a swim motor program, and synaptic connectivity (Willows et al., 1973b; Taghert and Willows, 1978; Getting et al., 1980; Hume and Getting, 1982; Getting, 1983b). The CPG interneurons (C2, DSI, and VSI-A) have their somata on the dorsal surface of the cerebral ganglion (Getting et al., 1980). The cell body of VSI-B is located beneath the ventral surface of the pleural ganglion (Getting, 1983b). The efferent flexion neurons (DFN-A,B and VFN) are located in the adjoining pedal ganglion (Hume et al., 1982). All of the neurons have contralateral homologs. The CPG interneurons project to the contralateral pedal ganglion (Getting et al., 1980), and it is thus likely that they synapse near the somata of the flexion neurons; however, none of the synapses have been anatomically located.

After identifying the neurons, the superfusion medium was switched to high divalent cation saline to reduce the contribution of polysynaptic pathways to any responses seen. All experiments were conducted in high divalent cation saline which consisted of (in mM): 285 NaCl, 10 KCl, 25 $CaCl_2$, 125 $MgCl_2$, 10 HEPES (pH 7.6), and 11 D-glucose. This high divalent cation saline is different from that used by other researchers in portions of previous studies (Getting, 1981; Hume and Getting, 1982; Getting, 1983b), and appears to be more effective at preventing polysynaptic contributions to synaptic potentials. As a result, the shapes of some of the monosynaptic potentials that we will refer to here (Table 1) differ from those previously reported due to the lack of polysynaptic contributions to the synaptic potentials. In particular, the C2 to VSI-B connection was reported to be excitatory (Getting, 1983b). In normal

saline, we also find that C2 excites VSI-B. However, in our high divalent cation saline, the monosynaptic potential is biphasic, with an initial small excitatory component followed by a prolonged and larger inhibitory component.

For all experiments where an accurate measure of membrane potential was required, the neuron being examined was impaled with two microelectrodes, one for current injection, the other for voltage recording. In other cases, current was injected through a balanced bridge circuit. Spikes in C2 and DSI were evoked using short depolarizing current pulses (5–20 msec). Each current pulse evoked a single spike, allowing the spike frequency to be precisely controlled. Data were recorded on VCR tape and analyzed off line. Portions of experiments were directly digitized using Cambridge Electronic Design hardware and software (SPIKE2). The digitization rate for intracellular recordings was 1KHz or greater. Traces produced from these recordings were sometimes signal averaged. Electrical noise was reduced through the use of a local-averaging routine on the digitized data, where consequtive 3–10 msec segments were averaged, and in some traces 60 Hz electrical noise was digitally subtracted.

For measurements of paired-pulse facilitation, preparations were excluded if the amplitude of the EPSP evoked by a single C2 spike was not resolvable. In some preparations, tonic depolarization of C2 with current injection allowed single spike-evoked EPSPs to be resolved. The amplitude of the first EPSP (EPSP1) was measured from its peak to the baseline initially preceding the spike. If the baseline was not level, then the amplitude was measured from a linearly extrapolated baseline. The amplitude of the second EPSP (EPSP2) was corrected for summation by measuring from its peak to the corresponding time on a single EPSP (see Fig. 8*A,B*). The amount of facilitation was quantified as the facilitation index (*Fi*), which was defined as the relative size of EPSP2 with respect to EPSP1:

$$Fi = \frac{\text{EPSP2} - \text{EPSP1}}{\text{EPSP1}}$$

Where applicable, two-tailed *t* tests were used to determine statistical significance. Results are expressed as the mean ± standard error of the mean.

Results

DSI enhanced the size of C2-evoked EPSPs in DFN-A

During a swim motor program, each of the six DSIs fires action potentials at rates over 20 Hz near the beginning of the swim and declining to 5–10 Hz by the end of the swim (Getting et al., 1980; Katz et al., 1994) (Fig. 1*B*). As previously shown (Katz et al., 1994), when a single DSI was stimulated to fire at a rate well within its natural spike frequency range for a swim, it caused the size of C2-evoked synaptic potentials recorded in the flexion neuron DFN-A to be enhanced two- to threefold (Fig. 2*A*). We sought to determine if this enhancement was due to presynaptic actions of DSI on C2 or postsynaptic effects of DSI on DFN-A.

Evidence against DSI acting at a postsynaptic site to enhance the size of C2-evoked PSPs

There were a number of potential postsynaptic mechanisms that could have contributed to the enhancement of the size of C2 synaptic potentials produced by DSI. These included changes in postsynaptic input resistance or a dependence of the C2-evoked EPSP on postsynaptic membrane potential. We tested for both possibilities and found that they did not play a role in the increasing the size of the C2-evoked EPSP.

Postsynaptic input resistance. If DSI stimulation increased the input resistance of DFN-A, it would cause the EPSPs evoked by C2 to become larger. Previous work reported a small increase in input resistance in DFN-A following DSI stimulation (Hume and Getting, 1982). However, in the present study, by using two electrodes in DFN-A and leveling the baseline by subtracting the EPSP waveform (Fig. 2*B,C*), we found no change in input resistance during the period following DSI stimulation ($N = 5$). This indicates that the increase in the size of C2-evoked EPSPs during this period is probably not due to a change in postsynaptic input resistance. However, we cannot eliminate the possibility that DSI caused a change in input resistance of DFN-A at a synaptic site too distant from our soma recording site to be detected.

Postsynaptic membrane potential. Another possibility is that the large increase in the amplitude of the C2 to DFN-A EPSP caused by DSI is due to a voltage-dependent process. This might occur through a voltage dependence of either postsynaptic mem-

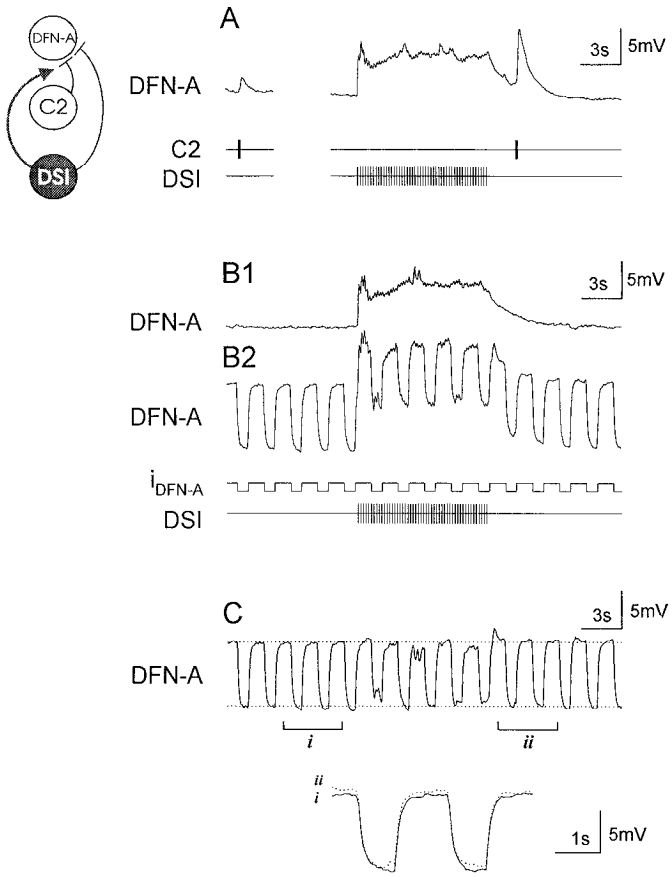

Figure 2. Enhancement of the size of the C2 to DFN-A EPSP by DSI was not associated with an increase in DFN-A input resistance. *A*, When C2 was stimulated alone (four pulses, 20 Hz), it evoked a small summated EPSP in DFN-A. Two seconds after DSI stimulation (5 Hz, 10 sec), the same C2 spike train evoked a much larger EPSP in DFN-A. *B*, The synaptic potentials evoked by a DSI train were not associated with a large change in input resistance in DFN-A. *B1*, DSI was stimulated alone (5 Hz, 10 sec) to evoke rapid, one-for-one EPSPs and a slowly decaying synaptic potential in DFN-A. *B2*, The above procedure was repeated while injecting hyperpolarizing current pulses into DFN-A. The changes in the amplitude of the voltage deflections due to DSI-evoked conductance changes were difficult to observe due to the shifting baseline. *C*, Subtraction of the DFN-A trace in *B1* from that in *B2* reveals the responses to current pulses without the shift in baseline caused by the DSI-evoked EPSPs. There was a slight decrease in input resistance during the DSI stimulus train. Immediately following DSI stimulation (*ii*), when C2-evoked EPSPs would be modulated, there is no difference in input resistance when compared to the beginning of the trace (*i*). The bottom traces are superimposed expansions of the two regions denoted by the *brackets*. In all cases, the prestimulus resting membrane potential of the DFN-A was −70 mV. This and all subsequent figures are from preparations superfused with high divalent cation saline.

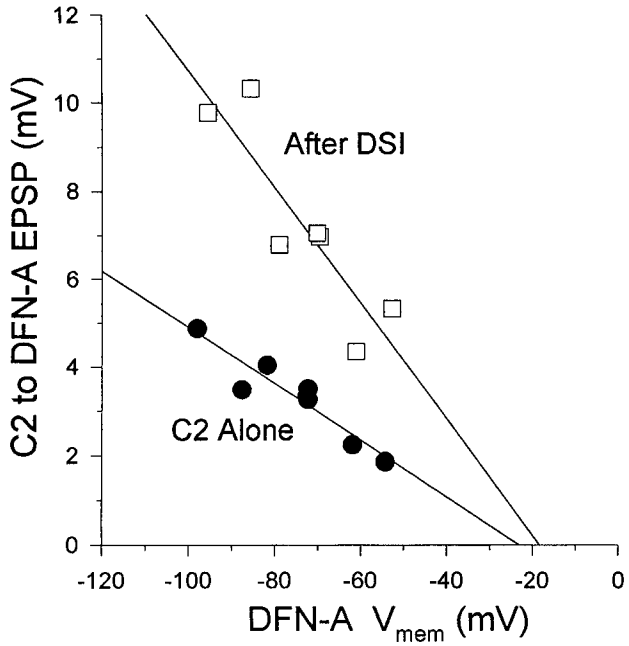

Figure 3. DSI enhancement of the size of C2 to DFN-A EPSPs did not exhibit voltage sensitivity. The initial membrane potential of DFN-A was altered by current injection through a second microelectrode in DFN-A. C2 was stimulated (four spikes at 20 Hz) either alone or 2 sec after a DSI train (5 Hz, 10 sec). The amplitude of the C2 to DFN-A EPSP increased as DFN-A was hyperpolarized due to an increase in driving force. DSI stimulation enhanced the amplitude of those EPSPs at all membrane potentials. The reversal potentials for the C2-evoked EPSPs when C2 was stimulated alone (*solid circles*) or when C2 was stimulated after DSI (*open squares*) were extrapolated using a linear regression least-squares fit to the data, and were not substantially different. The data are from a single preparation.

brane conductances or the transmitter-gated conductance itself. Alternatively, it might occur through a change in driving force caused by a simple depolarization if the C2 to DFN-A EPSP were due to a conductance decrease, or even through the secondary activation of another postsynaptic receptor. To determine if any of these mechanisms play a role in the enhancement of the size of C2-evoked PSPs by DSI, we varied the membrane potential of the postsynaptic target of C2 using a separate current injection electrode and measured the response to C2 stimulation alone and after DSI stimulation (Fig. 3).

The results showed that the increase in EPSP amplitude caused by DSI stimulation was not dependent upon the membrane potential of the DFN-A (N = 4), indicating that it was not due to a change in synaptic driving force or a voltage-dependent effect. In all four experiments, the percentage increase in the C2-evoked EPSP caused by DSI stimulation was not changed by altering the membrane potential of the DFN-A even though the driving force on the EPSP varied with membrane potential. If the DSI-evoked enhancement of synaptic potentials were caused by a change in driving force through a simple change in the postsynaptic membrane potential, then the enhanced EPSP amplitudes would have fallen along the same line as the control amplitudes, which they did not. If the modulation or the EPSP amplitude had depended on postsynaptic voltage, then either or both of the two plots in Figure 3 would not have been linear. Instead, we found that the data from all experiments were well fit by linear regressions.

Further evidence that the enhancement of the C2 to DFN-A EPSP amplitude was not due to a change in driving force or a change in the mixture of conductances underlying the EPSP is that the reversal potential of the EPSP was not affected by DSI stimulation. When C2 was stimulated alone, the extrapolated reversal potential of the EPSP evoked in DFN-A was -25.6 ± 5.9 mV. The reversal potential for the C2-evoked PSP did not change significantly following DSI stimulation, the average reversal potential being -24.6 ± 3.9 mV (N = 4) (for example, see Fig. 3). Instead what was observed was an increase in the slope of the relationship of EPSP amplitude to DFN-A membrane potential (Fig. 3), suggesting that DSI stimulation resulted in a larger synaptic conductance evoked by C2.

DSI differentially modulated synapses onto DFN-A

It was further expected that if DSI acted postsynaptically to enhance the size of the C2 to DFN-A EPSP, then the amplitudes of synaptic potentials evoked by other presynaptic neurons might also show an increase. We found that this was not the case. In fact, using the same stimulus paradigm that caused an increase in C2-evoked EPSPs (Fig. 4*A*), stimulation of one DSI decreased the amplitude of EPSPs evoked by other DSIs onto DFN-A (Fig. 4*B*). Single DSI-evoked EPSPs decreased an av-

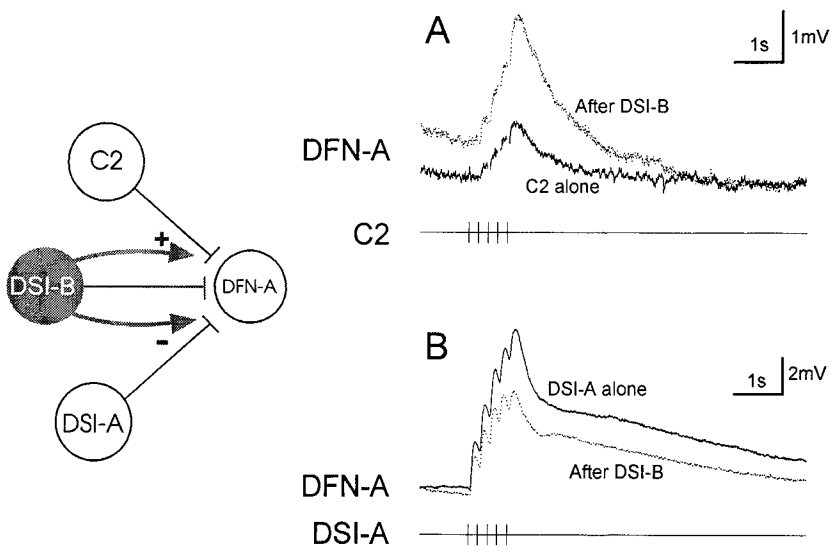

Figure 4. DSI stimulation differentially modulated inputs to DFN-A. *A,* C2 was stimulated to fire five spikes at 5 Hz either alone or 2 sec after a spike train in DSI-B (5 Hz, 10 sec). DSI-B stimulation increased the amplitude of the summated EPSP as well as the individual EPSPs. The pre-DSI stimulus DFN-A membrane potential was −66 mV for both traces. *B,* In the same preparation, DSI-A was stimulated in the same manner as C2: either alone or following a spike train in DSI-B. In this case, DSI-B stimulation caused a decrease in the amplitudes of the individual fast EPSPs evoked by DSI in DFN-A as well as in the summated slow EPSP. The prestimulus DFN-A membrane potential was −55 mV for both traces.

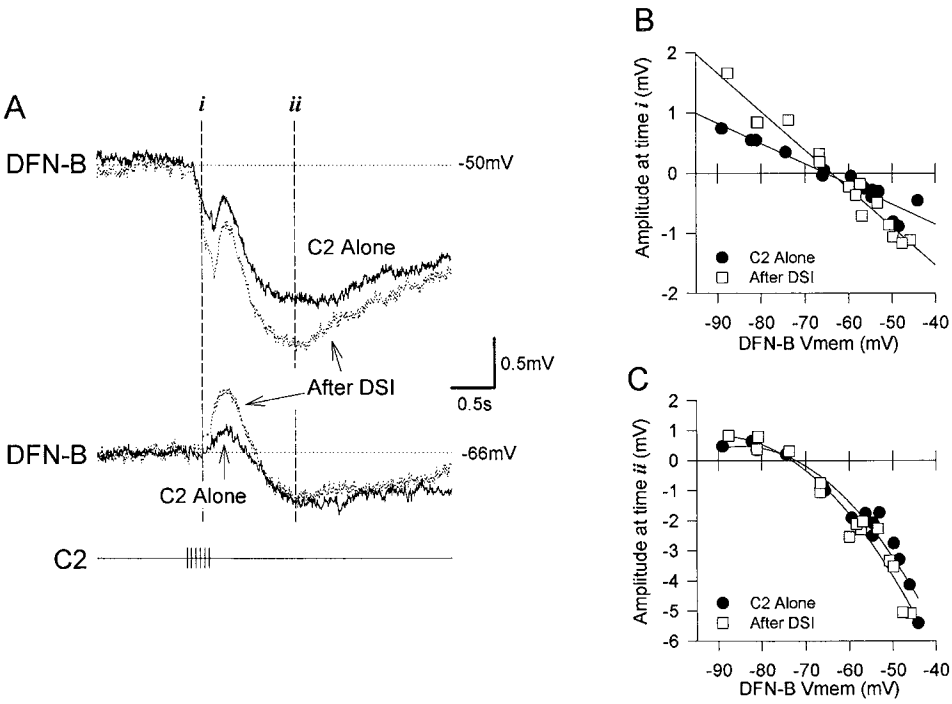

Figure 5. The amplitudes of all of the components of the C2 to DFN-B synaptic potential were enhanced by DSI stimulation. *A*, C2 stimulation (six spikes, 20 Hz) evoked a triphasic synaptic potential in DFN-B. At a resting potential of −50 mV (DFN-B, top traces), there was an initial inhibitory component followed by a depolarizing peak, and ending with a prolonged hyperpolarization. DSI stimulation (5 Hz, 6 sec), ending 4 sec before the C2 stimulation, appeared to increase the sizes all three components. At a resting potential of −66 mV (lower DFN-B traces), the initial inhibitory component was at its reversal potential and the size of the excitatory component was clearly enhanced by DSI stimulation. *B* and *C*, The amplitudes of the (*B*) initial inhibitory component (measured at the time shown by *i* in *A*) and (*C*) the slow inhibitory component (measured at the time shown by *ii* in *A*) plotted versus DFN-B membrane potential when C2 was stimulated alone (*solid circles*) and when C2 was stimulated after DSI (*open squares*). DSI stimulation enhanced the amplitude of the fast IPSP at all membrane potentials but did not change its reversal potential. DSI slightly enhanced the amplitude of the slow IPSP and also produced no change in its reversal potential.

erage of 34.4 ± 4.5% when preceded by stimulation of another DSI (5 Hz, 10 sec). The decrease was seen at all seven synapses examined in four different preparations and was statistically significant ($t = 3.74$, $P < 0.01$). This differential modulation by DSI of the synaptic potentials evoked by C2 and DSI suggests a presynaptic site for the modulation of at least one of these synapses.

DSI enhanced the amplitude of all of C2's chemical synapses in the swim network

We had previously examined the effects of DSI stimulation on C2-evoked synaptic potentials in other DSIs and in DFN-A (Katz et al., 1994). We extended this work to examine the effect of DSI stimulation at C2 connections onto all other known postsynaptic targets in the swim network. In addition to its connections with DSI and DFN-A, C2 also synapses with the ventral swim interneurons of the CPG (VSI-A, VSI-B) and with the other efferent flexion neurons (DFN-B and VFN) (Getting et al., 1980; Getting, 1981, 1983b; Hume and Getting, 1982). DSI stimulation enhanced the amplitude of C2 synaptic potentials recorded in each of these postsynaptic targets (Table 1).

Some of C2's monosynaptic effects are excitatory, others are inhibitory, and some are mixed excitatory/inhibitory (Getting, 1981; Snow, 1982b). DSI stimulation increased the amplitude of all of these monosynaptic connections, regardless of their sign (Table 1). This indicates that the modulatory effect is not specific for a certain type of postsynaptic receptor. For example, C2 evoked a triphasic response in DFN-B (Fig. 5*A*). There was an initial fast inhibitory component with a reversal potential of about −65 mV, which is near E_{Cl} for molluscan neurons (Kerkut and Meech, 1966; Ascher et al., 1976) (Fig. 5*A,B*). This fast inhibition was followed by a depolarizing potential (Fig. 5*A*). Finally, there was a prolonged inhibitory potential that rectified at potentials below about −75 mV or E_K (Kehoe, 1972) (Fig. 5*A,C*) as has been seen for other transmitter-evoked potassium conductances in mollusks (Katz and Levitan, 1993; Kehoe, 1994). At a resting potential of −50 mV, DSI stimulation (5 Hz, 6 sec) enhanced the size of both inhibitory components, but its effect on the excitatory component was obscured by the enlarged inhibitory components (Fig. 5*A*, top). However, by adjusting the membrane potential of DFN-B to E_{Cl}, the first inhibitory component could be nullified, revealing that DSI stimulation also enhanced the amplitude of the excitatory component (Fig. 5*A*, lower DFN-B trace). Similar results were obtained for all three C2 to DFN-B synapses examined.

DSI enhancement of C2 synaptic strength did not depend on the type of synapse made by DSI onto the target of C2

DSI itself synapses with many of the targets of C2 (Table 1). As with C2, the synaptic action of DSI at each of these synapses varies in sign. Regardless of whether DSI excited or inhibited the postsynaptic target of C2, it still increased the amplitude of synaptic potential evoked by C2 onto that target (Table 1). DSI even enhanced the size of C2 synaptic potentials at targets with which DSI itself did not synapse. For example, DSI did not evoke synaptic potentials in a Type IV neuron (Hume et al., 1982) (Fig. 6), yet it enhanced the size of the IPSP evoked by C2 in this cell (Fig. 6). This result again suggests that DSI pro-

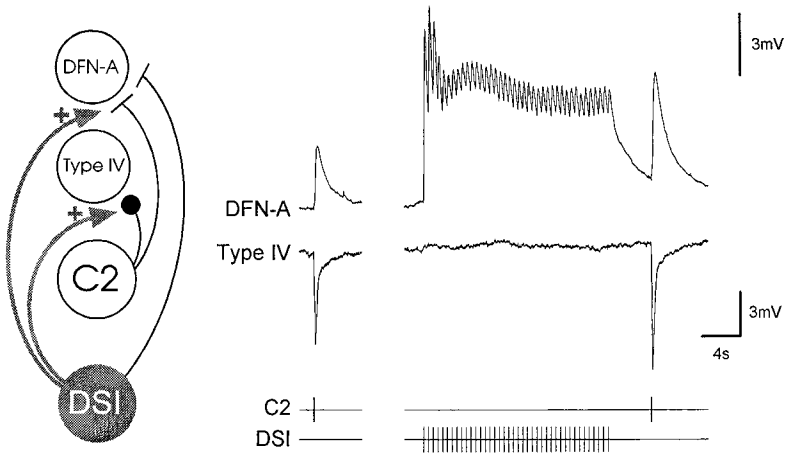

Figure 6. DSI enhanced the size of C2 synaptic potentials regardless of their sign or whether DSI itself synapses with that target. C2 stimulation evoked an EPSP in DFN-A and an IPSP in a Type IV neuron (Hume et al., 1982). DSI stimulation evoked one-for-one EPSPs in DFN-A, but had no conventional synaptic effect on the Type IV neuron. DSI stimulation enhanced the sizes of the C2-evoked EPSP onto both targets. The prestimulus membrane potential was −72 mV for the DFN-A and −38 mV for the Type IV neuron.

duces its modulatory effect by acting presynaptically on C2 rather than postsynaptically on each of the many target neurons of C2.

DSI stimulation did not enhance the strength of electrical coupling between the two contralateral C2s

While DSI strongly enhanced the strength of all of the chemical synapses made by C2, it had no effect on the electrotonic synapse made by C2 onto its contralateral homolog. We applied repeated hyperpolarizing current pulses to one C2 and measured the amplitude of the electrotonic potentials in its contralateral homolog during DSI stimulation. Although there was a small decrease in the electrically coupled potentials during DSI stimulation, probably due to shunting associated with the conventional EPSP evoked by DSI onto C2, no change in C2 to C2 electrical coupling was observed during the period following DSI stimulation, when the size of C2 chemical synapses was enhanced (N = 3, Fig. 7*A*).

To further test whether DSI stimulation affected C2-electrical coupling, we examined the electrotonic potentials produced by one C2 in the contralateral C2 while simultaneously monitoring the chemical synapse from C2 to DFN-A (Fig. 7*B*). Although the chemical EPSP recorded in DFN-A increased over two and a half fold in amplitude following DSI stimulation, the electrotonically coupled spikes in the contralateral C2 were almost completely unchanged from control (N = 2) (Fig. 7*B*).

DSI stimulation enhanced paired-pulse facilitation by C2: a presynaptic site of action

To conclusively determine if DSI enhancement of C2 synaptic strength was due to a presynaptic action on C2, we examined the effect of DSI on homosynaptic facilitation of C2 to DFN-A EPSPs using a paired-pulse protocol. Paired-pulse facilitation has been shown in other systems to be due to presynaptic mechanisms and has been attributed to calcium influx from the first spike increasing the amount of transmitter released by the second spike (del Castillo and Katz, 1954; Katz and Miledi, 1968; Sarrazin, 1987; Zucker, 1989; Wu and Saggau, 1994). The one way in which paired-pulse facilitation could be due to a postsynaptic mechanism would be if the amplitude of the EPSP was strongly voltage dependent. Since the C2-evoked EPSPs were not voltage dependent (see Fig. 3), any mechanism that affects facilitation must be acting on the presynaptic terminal.

We found that the C2-evoked EPSPs recorded in DFN-A exhibited paired-pulse facilitation (Fig. 8*A*). When C2 was stimulated alone, with a 100msec interval between spikes, the average facilitation index (*Fi*; see Materials and Methods) was 1.15 ± 0.88 (N = 3). If DSI acted purely postsynaptically, then the amplitudes of the first and second EPSPs would increase proportionally and *Fi* would remain unchanged. However, we found this not to be the case; in each of three preparations, following DSI stimulation (5 Hz, 10 sec), both the first and second C2 to DFN-A EPSPs increased in amplitude, but the size of the second EPSP increased by a larger percentage (Fig. 8*B*). As a result, *Fi* for a 100 msec interval increased to an average of 6.83 ± 3.08 (Fig. 8*C*). This change in facilitation indicates that DSI acts presynaptically to enhance the amount of transmitter released from C2.

DSI did not enhance transmitter release through a simple depolarization of C2

In many molluscan neurons, including C2, tonic depolarization by current injection can increase the size of spike-evoked synaptic potentials (Shimahara and Tauc, 1975; Shapiro et al., 1980; Getting, 1981; Snow, 1982b). This may be due to an effect on calcium conductances in the cell (Shapiro et al., 1980). Since DSI directly depolarizes C2 with both rapid and long-lasting EPSPs (Getting, 1981), it seemed possible that DSI might depolarize the terminals of C2 more strongly than the soma. We sought to determine if this depolarization could be responsible for the enhancement of C2-evoked synaptic potentials. If the DSI-evoked enhancement of synaptic release were due to a simple depolarization of C2, then depolarizing current injected into the soma of C2 ought to compete with the effects of DSI stimulation.

C2 was tonically depolarized to varying degrees with constant current injection. The amplitude of the C2-evoked synaptic potentials were proportional to the resting membrane potential of C2 at the time that the spikes were evoked (Fig. 9). Within a defined range of depolarizations, this relationship was linear.

In the six preparations tested, DSI stimulation (5 Hz, 10 sec) caused an increase in the amplitude of C2-evoked EPSPs regardless of the level of tonic depolarization of C2. The slopes of the relationships of C2-evoked PSP amplitude versus C2 membrane potential when C2 was stimulated alone and when C2 was stimulated following DSI stimulation indicate that DSI-evoked modulation of C2 synaptic potentials was not competitive with depolarization from current injection (Fig. 9*A*). If the DSI-evoked enhancement of C2 synaptic amplitude were due to depolarization of C2, then the two lines would be parallel; DSI

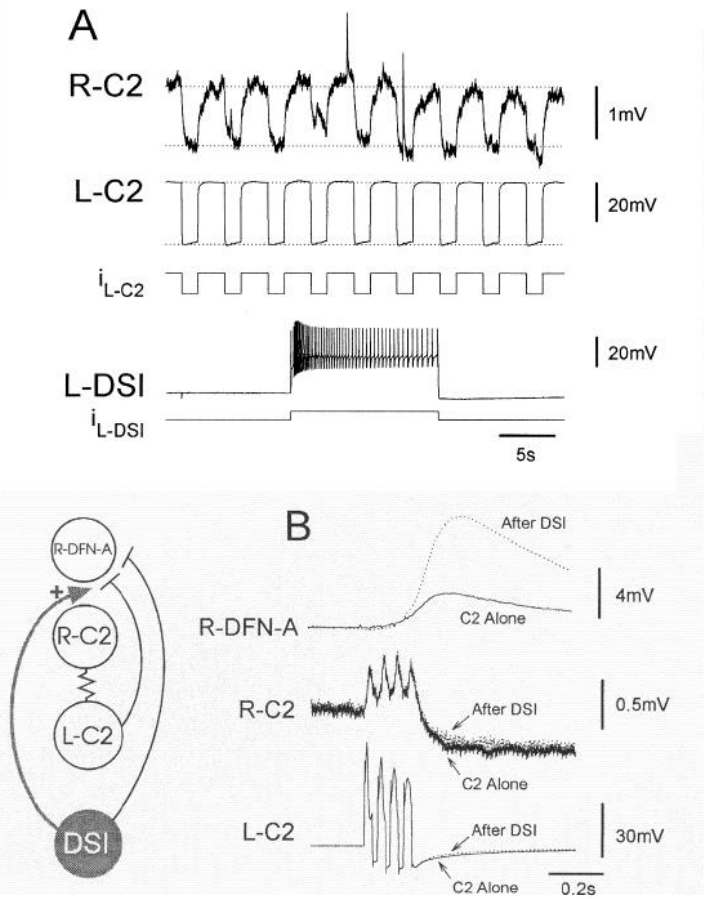

Figure 7. DSI did not enhance the strength of the electrical connection made by C2. *A,* The electrical coupling of the left C2 to the right C2 was tested by injecting repeated hyperpolarizing current pulses into the left C2. The amplitudes of the resulting voltage deflections in the right C2 are related to the input resistances of the two C2s and to the coupling resistance between them. DSI was injected with a step depolarizing current that caused DSI to fire at spike frequencies sufficient to enhance C2 synaptic strength. There was an initial decrease in the coupled potential recorded in the right C2. Following the DSI stimulation, there was no difference in electrical coupling. DSI makes synaptic connections only with the ipsilateral C2; therefore, it does not evoke one-for-one synaptic potentials in the contralateral C2 (Getting, 1981). A similar lack of change in the amplitude of the coupled potentials was observed when current positive pulses were used or when current was injected into the right C2. Prestimulus resting potentials: R-C2, −28 mV; L-C2, −29 mV; L-DSI, −41 mV. *B,* The electrotonically coupled spikes from the left C2 to the right C2 were not affected by DSI stimulation. C2 was stimulated with brief current pulses to fire four spikes at 20 Hz either alone (*solid traces*) or following DSI stimulation (*dotted traces*). The two sets of traces were overlaid. The four spikes in the left C2 resulted in four electrotonic potentials in the right C2 and an EPSP in the right DFN-A. DSI stimulation (5 Hz, 10 sec) caused a large increase in the size of the chemical EPSP evoked in DFN-A. No change was seen in the electrically coupled potentials in the R-C2 or in the spikes evoked in the left C2. Prestimulus resting potentials for both sets of traces: R-DFN-A, −70 mV; R-C2, −28 mV; L-C2, −22 mV.

would have caused a constant shift in the amount of depolarization needed to produce the various EPSP amplitudes. Instead, the two lines have different slopes, indicating that DSI uses a different mechanism than depolarization to enhance transmitter release from C2. Furthermore, current injection levels that saturated the depolarization-induced enhancement of C2-evoked EPSPs did not saturate the enhancement produced by DSI (not shown).

Discussion

The serotonergic DSIs are intrinsic components of the escape swim CPG circuit (Getting, 1981, 1989a), yet they also evoke neuromodulatory effects on other neurons in that same network; stimulation of a single DSI at physiological spike frequencies enhances the strength of synaptic actions by CPG neuron C2 (Katz et al., 1994). The results from this study show this synaptic enhancement is due to an increase in the amount of neurotransmitter released from C2 following DSI stimulation.

DSI enhancement of the size of C2 synaptic potentials is not due to postsynaptic mechanisms

Enhancement of C2-evoked synaptic potentials by DSI could have been caused by a presynaptic effect on C2, a postsynaptic effect on the targets of C2, or both pre- and postsynaptic actions. Although postsynaptic mechanisms have been shown to be involved in heterosynaptic enhancement of synaptic strength in other systems (Koerber and Mendell, 1991; Thompson et al., 1993), our results suggest that they are not playing any role in the enhancement caused by DSI. First, there is no significant change in postsynaptic input resistance to account for the two to three fold increase in synaptic strength. Second, the synaptic enhancement is not dependent upon the membrane potential of the postsynaptic neuron, and there is no change in the reversal potential of the C2-evoked PSPs.

If DSI enhanced the general responsiveness of the postsynaptic neuron, then the size of other inputs onto DFN-A might be expected to also be enhanced. Yet, we found that stimulation of one DSI decreases the amplitude of EPSPs evoked by other DSIs onto DFN-A. This by itself does not prove that DSI is acting presynaptically on both cells; we do not yet know if the effect on the synapses from other DSIs is presynaptic.

Although unlikely, it might also be the case that there is a specific increase in the responsiveness of DFN-A to the neurotransmitter released by C2. C2 is peptidergic and is immunoreactive for SCP_B and FMRFamide (Snow, 1982a; Longley and Willows, 1985). A large molecular weight compound isolated from C2 mimics the multiphasic synaptic potentials onto at least one of its postsynaptic followers (Snow, 1982a). The identity of this compound is not yet known so we could not readily test whether DSI stimulation alters the responses to this substance. However, DSI stimulation does enhance the response of each postsynaptic target of C2 regardless of the sign of the synaptic response. These different responses, including multiphasic responses, are likely to be mediated by separate receptors. It seems unlikely that DSI would enhance the responses of each of those different receptors.

A further suggestion that DSI acts on C2 and not the postsynaptic followers of C2 is that DSI enhances the strength of C2 connections onto cells that DSI itself does not synapse with. While it is possible that DSI releases 5-HT in the vicinity of these cells and that these cells merely lack receptors which would produce voltage changes, the most parsimonious explanation is that DSI acts on C2 itself.

DSI acts presynaptically to enhance transmitter release from C2

The previously mentioned evidence only suggests that DSI acts to enhance transmitter release from C2. Due to the unknown anatomical organization of the synaptic sites, we chose not to attempt a quantal analysis to determine whether the action is presynaptic as has been done for other systems (Korn and Faber,

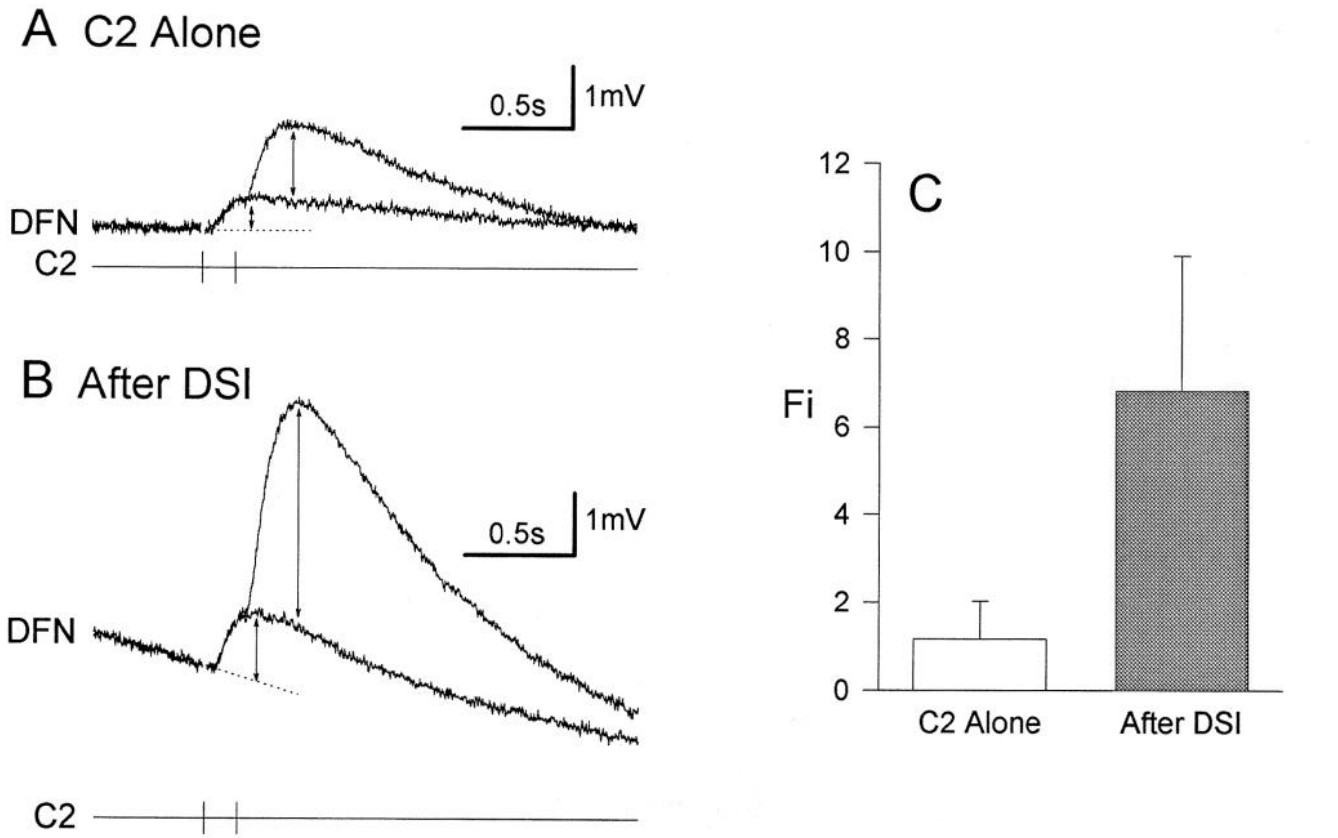

Figure 8. DSI enhancement of the C2 to DFN-A EPSP amplitude was associated with an increase in C2 paired-pulse facilitation. *A,* When C2 was stimulated alone, it exhibited paired-pulse facilitation. Here, C2 was stimulated to fire two spikes 150 msec apart, evoking a pair of EPSPs in DFN-A. C2 was also made to fire a single spike, resulting in a unitary EPSP. The two traces were overlaid. The second EPSP was measured from its peak to the corresponding point on the first EPSP. The second EPSP was larger than the first, indicating facilitation. *B,* Two seconds after DSI stimulation (5 Hz, 10 sec), the sizes of both EPSPs evoked by C2 were enhanced. The EPSPs are riding on the tail end of the slow component of the DSI to DFN-A EPSP. The first EPSP was measured from its peak to the linearly extrapolated baseline. The second EPSP was measured as in *A*. The amplitude of the second EPSP was enhanced to a greater extent than that of the first EPSP, indicating an increase in the amount of paired-pulse facilitation. The prestimulus resting potential for the DFN-A in both *A* and *B* was −70 mV. *C,* The facilitation index, *Fi*, which is a measure of the relative size of the second EPSP with respect to the first (see Materials and Methods), was calculated from three preparations with C2 interspike intervals of 100 msec. When C2 was stimulated alone, the average *Fi* was 1.15 ± 0.88. Following DSI stimulation, *Fi* increased to 6.83 ± 3.08.

1991; Mintz and Korn, 1991; Lisman and Harris, 1993). The strongest direct evidence that DSI acts presynaptically on C2 is that it affects the homosynaptic facilitation of C2-evoked EPSPs. Facilitation, measured using a paired-pulse paradigm, has been shown to be caused by a presynaptic mechanism where calcium entering the presynaptic terminal during the first spike enhances the release of neurotransmitter by the second spike (del Castillo and Katz, 1954; Katz and Miledi, 1968; Sarrazin, 1987; Zucker, 1989; Wu and Saggau, 1994). Anything that alters the amount of facilitation must be interfering with either calcium dynamics in the presynaptic terminal or calcium-triggered release.

Other studies have used alteration of paired-pulse facilitation as proof of a presynaptic locus for synaptic changes (Manabe et al., 1993; Christie and Abraham, 1994; Leung and Fu, 1994; Schulz et al., 1994). Usually, an enhancement of synaptic efficacy is associated with a decrease in facilitation. This is interpreted to indicate that the enhancement occurs through an increase in calcium entry, thus competing with the facilitation mechanism (Kumamoto, 1991). However, under certain conditions, synaptic enhancement has been associated with an increase in facilitation (Schulz et al., 1994). In this case, the mechanism underlying the enhancement is separate from that underlying facilitation, perhaps involving a direct effect on the release mechanism itself.

While we do not yet know the mechanism underlying DSI enhancement of C2 synaptic potentials, we do know that DSI acts specifically on the chemical synapses of C2; it has no effect on the electrical connection made by C2. We have also shown that the enhancement of the chemical synapses is not due to a simple depolarization of C2. Thus a modulatory action directly affecting the release mechanism is possible.

Enhancement of neurotransmitter release in other systems

The DSIs are serotonergic (Katz and Frost, 1993; Katz et al., 1994; McClellan et al., 1994) and 5-HT has been shown to enhance the release of neurotransmitter in other systems. Two of the most studied examples are the effects of 5-HT on sensorimotor synapses in *Aplysia* (Kandel and Schwartz, 1982; Byrne et al., 1991; Klein, 1994) and crustacean neuromuscular synapses (Enyeart, 1981; Kravitz et al., 1981; Glusman and Kravitz, 1983; Delaney et al., 1991). In addition, 5-HT enhances the release of glycine from inhibitory synapses onto Mauthner cells (Mintz and Korn, 1991). Other neuromodulatory substances have also been shown to enhance release of neurotransmitters

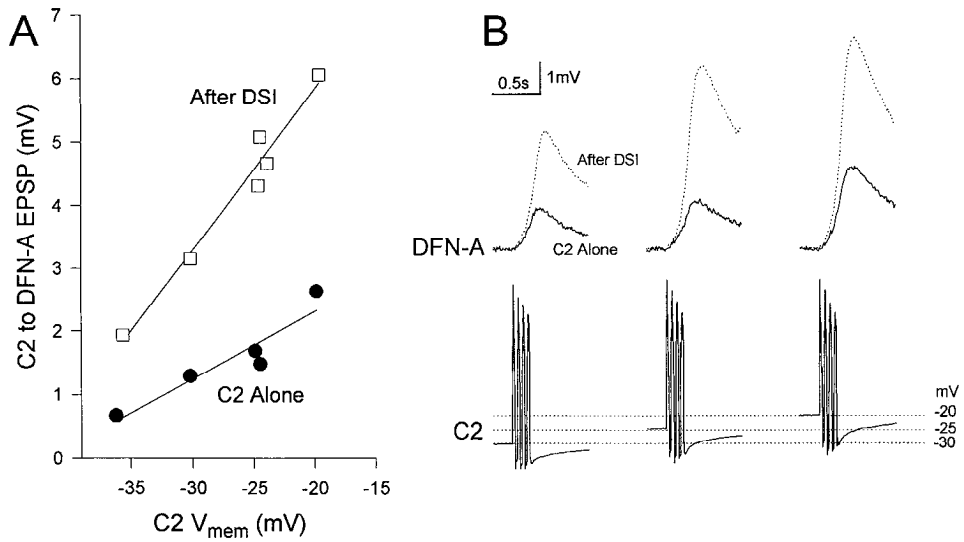

Figure 9. DSI enhancement of the size of C2-evoked EPSPs was not caused by a simple depolarization of C2. As seen with other molluscan neurons, the amplitude of C2-evoked synaptic potentials was dependent upon the resting membrane potential of the presynaptic neuron. Varying levels of current were injected into C2 through a second electrode, changing its resting membrane potential. The amplitude of EPSPs evoked by spikes in C2 increased with increasing depolarization of C2. *A,* C2 was stimulated alone (*solid circles*) or 2 sec after DSI stimulation (*open squares*). DSI stimulation (5 Hz, 10 sec) increased the amplitude of C2-evoked EPSPs at all C2 membrane potentials. If DSI enhancement of C2-evoked EPSPs were caused by depolarization of C2, then the two curves would be parallel. Instead, they have different slopes. *B,* Sample traces showing C2 spikes and the resulting EPSPs in DFN-A when C2 was stimulated alone (*solid traces*), or following DSI stimulation (*dotted traces*). The C2 resting membrane potential for each pulse train is shown by the horizontal dotted lines. C2 spikes were elicited by four 20 msec constant current pulses from the various resting potentials. The prestimulus resting potential of the DFN-A was −70 mV for all traces.

(Langer, 1980; Libet, 1986; Chesselet, 1984; Starke et al., 1989; Wonnacott et al., 1989). For example, dopamine increases synaptic release from GABAergic neurons in the striatum (Cameron and Williams, 1993). In another CPG network, the stomatogastric nervous system, the peptide red pigment concentrating hormone (RPCH) and the biogenic amine octopamine appear to enhance release at some synapses, thereby altering the production of motor patterns (Dickinson, 1989; Johnson and Harris-Warrick, 1990; Dickinson et al., 1993).

Despite the many demonstrations of chemically induced presynaptic enhancement of release, to our knowledge, the present study represents the first example of an individual neuron directly enhancing the release of neurotransmitter from an interneuron. In *Aplysia*, heterosynaptic facilitation has been demonstrated at a number of sensorimotor synapses in response to stimulation of modulatory neurons (Hawkins et al., 1981; Hawkins and Schacher, 1989; Mackey et al., 1989), but despite some suggestive results (Shimahara and Tauc, 1976), there has been no conclusive demonstration of this effect at interneuronal synapses in *Aplysia*. Evidence also strongly suggests that serotonergic enhancement of transmitter release demonstrated at synapses onto Mauthner cells in goldfish is likely to be caused by local 5-HT neurons (Mintz et al., 1989; Mintz and Korn, 1991). Furthermore, the vast literature showing the localization of the synaptic terminals of neurons containing modulatory substances at sites affected by those same neuromodulatory substances indicates that heterosynaptic enhancement of transmitter release is likely to be very important for many interneuronal circuits.

The function of intrinsic neuromodulation in the Tritonia *swim network*

The enhancement of neurotransmitter release from C2 by DSI is likely to be important for the *Tritonia* swim circuit, particularly for network reconfiguration. The swim network exists in at least two configurations: a single-shot reflexive withdrawal circuit and a rhythmic swim CPG circuit (Getting, 1977; Getting and Dekin, 1985a; Getting, 1989a,b). It has been suggested that the C2 neuron is important for the reconfiguration of the network into the oscillatory state (Getting and Dekin, 1985a,b), yet, in the rested state, before a swim has occurred, the synaptic connections made by C2 onto its CPG targets are all relatively weak and ineffective (unpublished data). Furthermore, C2 excitability prior to a swim is low (Frost et al., 1988). DSI activity rapidly enhances both C2's synaptic efficacy and its excitability (Katz and Frost, 1994). In the rested state, the DSIs exhibit a low rate of spontaneous firing, not enough to produce an enhancement of C2 synapses. When a swim stimulus arrives, the DSI neurons fire very rapidly. By quickly enhancing the strength of C2's connections and its excitability, DSI activity may functionally rewire the network and transform it so that C2 is in a position where it is able to evoke widespread effects, thus enabling the reconfiguration to occur. By acting presynaptically to enhance transmitter release from C2 instead of postsynaptically to enhance responsiveness, DSI is able to preferentially enhance only the connections of C2. In fact, DSI decreases the connections strengths of other DSI neurons onto those same targets. Thus, DSI alters the balance of synaptic inputs to various targets. By enhancing transmitter release from specific synapses, intrinsic neuromodulation may participate in the transient "crystallization" of a functional neuronal circuit out of a diffuse network of anatomically connected neurons.

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