Selective Serotonin Reuptake Inhibitors Induce Spontaneous Interneuronal Activity in the Leech Nervous System

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INTRODUCTION

Serotonin [5-hydroxytryptamine (5-HT)] is a conspicuous neuromodulator of sensory–motor networks (Jacobs and Fornal 1993; Schmidt and Jordan 2000) that acts at different levels of the motor system hierarchy (Harris-Warrick and Marder 1991; Jankowska 2001; Schmidt and Jordan 2000).

Understanding the mechanism of action of 5-HT on any given network represents a major challenge because of the multiplicity of action sites and the broad variety of 5-HT receptors (Barnes and Sharp 1999). Given the variety of its actions on sensory–motor networks, serotonin can affect a variety of neurons at different levels of the network hierarchy. Because of its many possible targets, it has been difficult to obtain a comprehensive picture of how serotonin affects the nervous system by means of a selective serotonin reuptake blocker (SSRI), fluoxetine. Fluoxetine evoked barrages of synaptic potentials in identified sensory, motor, and interneurons. This effect was mimicked by the tricyclic antidepressants imipramine and clomipramine, and by the SSRI citalopram, with relative efficacies that matched their known relative selectivities for the 5-HT transporter. The synaptic responses evoked by fluoxetine in different neurons were temporally correlated, suggesting that they had a common origin. The profile of the synaptic responses matched that expected from the activation of the mechanosensory pressure cells, known to act by paracrine and synaptic effects (Kristan and Nusbaum 1983). Serotonin also modulates the leech shortening response (Wittenberg and Kristan 1992a) and its plasticity (Belardetti et al. 1982).

The prevailing strategy in these investigations was to expose the nervous tissue to exogenous 5-HT. Given the presence of different 5-HT receptors that evoke opposite actions on leech neurons (Acosta-Urquidi et al. 1989; Sanchez-Armass et al. 1991), in leeches (Bruns et al. 1993; Henderson 1983), and in other organisms (Barbas et al. 2003; Teshiba et al. 2001), it is important to study the effects produced by 5-HT released from endogenous sites. Our hypothesis is that proximity between 5-HT release sites and specific targets could be determinative in the modulatory outcome (see Teshiba et al. 2001).

The aim of the present study was to analyze the effects of 5-HT released from endogenous sources on sensory, motor, and interneurons and compare them with those evoked by bath-applied 5-HT. Based on the effectiveness of 5-HT reuptake inhibitors in the leech (Bruns et al. 1993; Henderson 1983), we used the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Wong et al. 1995) to cause the accumulation of 5-HT extracellularly. Our results suggest that endogenously released 5-HT caused the activation of an interneuronal layer that spans the nerve cord, eliciting responses that resemble those evoked by mechanosensory stimulation, and different from those evoked by bath-applied 5-HT.

METHODS

Biological preparation

Leeches, Hirudo medicinalis, weighing 2–5 g, were obtained from a commercial supplier (Leeches USA, Westbury, NY) and maintained analyzing its effects at different sites of the network hierarchy. The nervous system of the leech Hirudo medicinalis offers unique experimental advantages for such study because identified sensory, motor, and interneurons can be recorded simultaneously.

In the leech, serotonin increases the probability of producing the swim motor pattern (Hashemzadeh-Gargari and Friesen 1989; Willard 1981). This monoamine altered the physiological properties of swim-initiating neurons (Angstadt and Friesen 1993a,b), motoneurons (Mangan et al. 1994a,b), and muscle fibers (Mason and Kristan 1982), favoring the induction of swimming. It has been suggested that 5-HT induces swimming, acting by paracrine and synaptic effects (Kristan and Nusbaum 1983). Serotonin also modulates the leech shortening response (Wittenberg and Kristan 1992a) and its plasticity (Belardetti et al. 1982).

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at 15°C in artificial pond water. The animals were not fed for at least 1 mo before dissection. The leech nervous system is composed of a chain of 21 midbody ganglia between the head and tail brains. Matching the similarity between the segments they innervate, the midbody ganglia are almost indistinguishable from one another. Each one bears a full set of sensory and motor neurons, whereas the interneurons show 3 different configurations: 1) neurons that are confined within each ganglion; 2) neurons that have their somata in one ganglion and extend arborizations toward several anterior or posterior ganglia; or 3) descending interneurons from either of the 2 brains. It is worth noting that when a ganglion is isolated, the interneuronal layer of sensory–motor networks remains functional in spite of the fact that the interneurons could be detached from their somata.

Individual midbody ganglia—or chains of ganglia, where stated—were dissected out of the animal and pinned ventral side up to the Sylgard (Dow Corning, Midland, MI) base of a superfusion chamber. Preparations were continuously superfused with physiological saline at room temperature. The sheath covering the ganglion was dissected out, leaving the neuronal cell bodies directly exposed to the external solution.

In the experiments described in Fig. 7, the preparation consisted of a chain of 5 ganglia in a split-bath chamber. In this recording configuration, 2 ganglia of the chain were surrounded by a Vaseline well to form a waterproof seal that separated them chemically from the other 3 ganglia. The solution in the Vaseline well was under constant superfusion, whereas the rest of the chamber was maintained continuously in physiological saline. The advantage of this preparation is that ganglia in each compartment can be exposed to saline solutions with different compositions while their anatomical connections are preserved intact (Willard 1981).

The experiments were carried out between February 2002 and May 2004. We excluded experiments performed from July to December 2002 because during this period no effect of fluoxetine was observed in the studied ganglia (n = 33). To evaluate possible seasonal variation in endogenous 5-HT (Catarsi et al. 1990), we analyzed the 5-HT content in chains of ganglia 2 to 20. Serotonin content was measured by high-performance liquid chromatography (Gilson S.A.S., Villiers Le Bel, France), using a 5-μm UltraspHERE ODS column (4.6 × 250 mm; Beckman Coulter, Fullerton, CA) and detected amperometrically with an oxidation potential of +0.70 V (Model 141, Gilson). We found a substantially lower 5-HT content (11.93 ± 3.5 pmol/ganglion, n = 2) during the fluoxetine-insensitive period, than that during periods of normal activity (26.72 ± 5.82 pmol/ganglion, n = 5). However, this phenomenon was not observed again.

Solutions

The physiological saline composition was as follows (in mM): NaCl, 115; KCl, 4; CaCl₂, 1.8; MgCl₂, 1; Tris base, 5.4; pH 7.4. To block synaptic transmission, we used a solution with a high Mg²⁺/Ca²⁺ ratio (7 mM MgCl₂/1 mM CaCl₂), in which the osmolarity was kept constant by reducing the NaCl concentration. We chose a concentration of 7 mM Mg²⁺, rather than the classically used 20 mM (Baylor and Nicholls 1969), to preserve the excitability of the neurons. To test the effectiveness of different Mg²⁺ concentrations, we studied the interaction between the mechanosensory P cell (sensitive to pressure) and the annulus erectore (AE) motoneuron (Isca et al. 1999). In the presence of 7 mM Mg²⁺/1 mM Ca²⁺, the P–AE interaction was completely and reversibly abolished (n = 5), although both neurons retained their normal excitability on direct electrical stimulation.

The different drugs used in this study were dissolved in physiological saline and applied through superfusion at a rate of 0.3 ml/min. The exchange of solutions was performed using a solenoid-operated solution switcher (Valve driver II, General Valve, Fairfield, NJ). In our superfusion system it takes approximately 45 s to reach a complete exchange of the external solution (Szczupak et al. 1993). In some experiments, 5-HT was ejected onto the soma from a micropipette filled with a saline solution containing 1 mM 5-HT, by applying pressure pulses using a Picospritzer (General Valve).

The concentration of 50–100 μM of the 5-HT reuptake blocker is in the same range as that used in other studies in invertebrates in which the drug is applied to the whole nervous system (Katz and Frost 1995), rather than to isolated neurons (Henderson 1983; Ranganathan et al. 2000).

5-Hydroxytryptamine (serotonin), fluoroxetine, imipramine, and clonopram mesylate were purchased from Sigma-Aldrich (St. Louis, MO). Clomipramine was purchased from RBI (Natick, MA). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) was purchased from Sigma-Genosys (The Woodlands, TX).

Electrophysiological recordings

Neuronal activity was recorded using intracellular glass microelectrodes connected to an amplifier Axoclamp 2B (Axon Instruments, Foster City, CA), operating in current-clamp configuration. Microelectrodes were pulled from borosilicate capillary tubing (FHC, Brunswick, ME) and filled with a 3 M potassium acetate solution. Electrodes with a resistance of 30–40 MΩ were selected. The recordings were digitized using a Digidata 1322A interface and acquired using Clampex protocols (pClamp 8.0.2, Axon Instruments) at sampling frequencies of 2 kHz. Neurons were identified by their location, size, and electrophysiological properties (Muller et al. 1981).

Data analysis

The recordings were analyzed using commercial software (Axograph 4.5, Axon Instruments). The results are reported as means ± SE and the number of independent observations is expressed between parentheses (n). Statistical significance of the results obtained in different experimental conditions was determined by Student’s t-test. Curve fitting was achieved using a commercial software (Kaleida- graph 3.0.2, Abelbeck Software, Reading, PA).

To perform the cross-correlation analysis, the spikes in the AE recordings were low-pass filtered at 2–7 Hz. The filtered AE recordings were cross-correlated with the paired Retzius (Rz) neuron recordings, using a bin size of 12.5 ms.

Results

Effect of the SSRI on the electrophysiological activity of neurons in isolated leech ganglia

The long-term goal of the present series of experiments is to study how endogenously released 5-HT affects the electrophysiological activity of sensory–motor networks in the leech nervous system (briefly described in METHODS). Two independent studies, performed by Henderson (1983) and Bruns and collaborators (1993), showed that 5-HT reuptake blockers, used in mammals, effectively inhibited 5-HT reuptake in the leech nervous system. In addition, they showed that blockade of 5-HT reuptake increases the synaptic responses evoked by serotonergic neurons. Based on this information, we analyzed the effects of the SSRI fluoxetine (Wong et al. 1995) on the electrophysiological activity of specific neurons in the isolated midbody ganglion.

Two neurons were initially chosen to monitor the activity evoked by fluoxetine: the AE motoneuron, responsible for erection of the annuli that subdivide the leech skin (Rodriguez et al. 2004; Stuart 1970), and the Retzius (Rz) neuron, the main
5-HT source in the leech nervous system (McAdoo and Coggeshall 1976). There is one pair of AE and one pair of Rz neurons in each midbody ganglion and these 2 types of neurons do not bear any kind of synaptic interactions between them.

Simultaneous recordings of these cells show that 50–100 μM fluoxetine evoked barrages of spontaneous synaptic activity in both neurons (n = 38 out of 43 ganglia). In the context of the present report, “spontaneous” means without any other experimental stimulation but the presence of the chemical agent at test. Figure 1 exhibits representative recordings in which the Rz neuron displays spontaneous bursts of action potentials surmounted on excitatory synaptic potentials (EPSPs), whereas the AE neuron displays inhibitory synaptic potentials (IPSPs). Whereas in control conditions Rz neurons fired at a steady frequency, the beginning of the fluoxetine effect was marked by small bursts of, at least, 2 to 3 spikes that coincided with IPSPs in the AE recording. In this way, the onset of the fluoxetine effect was defined in the Rz recordings based on the marked change in its firing regime: in average, the firing frequency of the Rz (n = 38) neurons in control solution was 0.22 ± 0.03 Hz, whereas during the initial bursts evoked by fluoxetine the frequency was 13.13 ± 2.39 Hz. This marked the initiation of a period of intense synaptic activity in both neurons, whose average delay, after switching the superfusion from normal saline to the one containing the SSRI, was about 2.7 min (2.75 ± 0.23 min). Subtracting the lag inherent to the superfusion system (see METHODS), the actual delay was about 2 min.

The prolonged latency to onset of the fluoxetine effect suggests that it is produced, indirectly, by causing the accumulation of endogenous 5-HT in the extracellular space. However, to rule out a possible direct effect of fluoxetine on the studied neurons, we analyzed the effect of fluoxetine in the presence of a high Mg²⁺/Ca²⁺ ratio that prevents neurotransmitter release in the leech (see METHODS). In this condition, fluoxetine did not produce any recordable effect on the electrophysiological activity of the Rz and AE neurons (n = 4; data not shown).

It is noteworthy that, although the synaptic responses of both neurons were of opposite sign, the synaptic events exhibited a marked temporal coincidence. The inset in Fig. 1 shows an expanded view of a recording fragment in which it is possible to appreciate that, although the EPSPs in Rz and the IPSPs in AE could exhibit different durations, they had a similar onset. To evaluate the degree of coincidence between the responses of Rz and AE neurons, a cross-correlation analysis (see METHODS) was implemented using recording segments (60 s) within the most active period of the SSRI effect. We analyzed 14 different paired recordings in which fluoxetine produced strong responses in both neurons. We assume that this selection did not bias the analysis, but the opposite: the more active the pair, the less they should be correlated if the correlation was not inherent to the phenomenon under study. Figure 2 shows the average cross-correlogram that indicates that the Rz EPSPs and the AE IPSPs in the selected recordings were highly coincident (cross-correlation index of −0.46 ± 0.08). In other recordings,
we encountered that the IPSPs in AE were of very low amplitude ($n$/H11005), the Rz neurons presented EPSPs of low amplitude and the action potential bursts were composed of a few spikes ($n$/H11005). All these cases ($n$/H11005) were not included in the cross-correlation analysis, but visual inspection of these recordings always showed a clear temporal correlation between EPSPs and IPSPs.

The spontaneous synaptic activity lasted for nearly 3.5 min (3.46 ± 0.27 min). At the end of this period, the cells returned to baseline. However, in 20% of the studied ganglia, exposure to fluoxetine produced a final steady hyperpolarization in AE.

The pharmacological profile of the fluoxetine effects

To evaluate whether fluoxetine actions were the result of its effect as an SSRI, we tested the effects of other 5-HT reuptake inhibitors. On one hand, we chose a pair of tricyclic antidepressant drugs, imipramine and clomipramine, that are less selective than fluoxetine when comparing their affinity to serotonin versus noradrenaline or dopamine transporters (Wong et al. 1995). On the other hand, we tested the effects of citalopram, a highly selective 5-HT reuptake inhibitor (Popik 1999; Stanford 1996).

The experiments followed a similar protocol to that described in Fig. 1. Clomipramine ($n$/H11005) or imipramine ($n$/H11005) evoked spontaneous synaptic events in Rz and AE neurons that were qualitatively similar to those evoked by fluoxetine, although these events were sparser. Figure 3A shows a representative example of the effect elicited by imipramine during its more active period. The Rz and AE neurons showed correlated EPSPs and IPSPs, respectively, but the events occurred at a much lower frequency than those evoked by fluoxetine. Rz neurons exhibited depolarizations that resembled plateau potentials, but the characteristics of these responses were not analyzed. On the other hand, superfusion with 50 μM ($n$/H11005) and 100 μM ($n$/H11005) citalopram evoked responses that were similar to those produced by fluoxetine but had a faster onset and lasted for a longer period. Figure 3B shows representative recordings of Rz and AE neurons evidencing a highly correlated occurrence of EPSPs and IPSPs. The synaptic events induced by citalopram (100 μM) started 1.67 ± 0.33 min after the superfusion was initiated (an actual delay of 57 s), and lasted for ≥13.33 ± 1.33 min. This duration was significantly longer than that measured for fluoxetine ($P < 0.05$).

These results indicate that inhibitors of 5-HT reuptake of different pharmacological nature evoked similar physiological effects, supporting the notion that the spontaneous synaptic activity induced by them was attributed to their common effect, the inhibition of 5-HT reuptake.

Profile of neuronal responses in the leech ganglia to the SSRI

The aim of the following series of experiments was to investigate the responses evoked by fluoxetine in other identified neurons.

We extended the study of the fluoxetine effect on the following leech neurons: motoneurons innervating circular muscles [circular ventral excitor (CV) cell, $n$ = 4] that are...
active during the elongation phase of the crawling motor pattern (Baader 1997; Eisenhart et al. 2000); the anteropagoda motoneuron-like cells (AP cell, \( n = 4 \)); the premotor nonspiking neurons (NS cell, \( n = 3 \)) that have been shown to regulate sensory–motor interactions (Iscla et al. 1999) and coactivation among motoneurons (Rela and Szczupak 2002); the swim-initiating neurons 204 and 61 (\( n = 2 \) for each type); and the S interneurons (\( n = 4 \)) that form a fast conducting pathway through the nerve cord (Frank et al. 1975) and play an important role in shortening plasticity (Sahley et al. 1994). We studied the effect of fluoxetine on each one of these neurons using the intracellular recording of one Rz neuron to monitor the temporal correlation of the responses evoked by the SSRI (Fig. 4). All these neurons, except the S cell, exhibited spontaneous excitatory inputs that were temporally correlated with those in the simultaneously recorded Rz neuron.

The response of the S cell to fluoxetine was somewhat more complex (Fig. 5). Fluoxetine caused a substantial steady increase in the S cell firing that was maintained throughout the fluoxetine superfusion period. In addition, the S cell exhibited a barrage of IPSPs coincident with the spontaneous Rz excitatory inputs.

The remarkable temporal correlation of the synaptic events evoked by fluoxetine in all the studied neurons suggests that this SSRI caused the excitation of a common afferent pathway. Examination of previous work indicates that the synaptic responses elicited in the different neurons resemble those generated by the excitation of the mechanosensory P cells, through polysynaptic pathways. Stimulation of P cells causes EPSPs in Rz cells (Wittenberg et al. 1990), AP cells (Wessel et al. 1999), NS cells (previously known as cell 151) (Marin Burgin and Szczupak 2000), and cells 204 (Debski and Friesen 1987); and IPSPs in AE cells (Iscla et al. 1999) and S cells (Wittenberg and Kristan 1992b).

These observations suggest that the widespread actions elicited by endogenous 5-HT on the leech nervous system could have been caused through the excitation of 2 main targets: 1) the mechanosensory P neurons or 2) an interneuronal layer that conveys the mechanosensory signals.

**Effects of fluoxetine on the electrophysiological activity of the mechanosensory neurons**

Leeches detect mechanical signals applied to their external surface through 3 distinct mechanosensory neurons present in each midbody ganglion. Cells T, P, and N are sensitive to light touch, pressure, and noxious stimuli, respectively, and each ganglion has 3 pairs of T cells and 2 pairs of N and P cells.

Superfusion of ganglia with fluoxetine did not evoke any change in the activity of P and N cells (\( n = 5 \) and 6, respectively). Figure 6A shows representative dual recordings of Rz and P neurons, revealing that whereas the Rz neuron responded with a barrage of EPSPs, the P cell remained silent. However, fluoxetine evoked breggves of IPSPs in the T cells (\( n = 4 \)). Figure 6B shows simultaneous recordings of Rz and T neurons, illustrating that both cells exhibited correlated synaptic activity of the opposite sign.

Because T cells receive inhibitory signals from P and N cells through a polysynaptic pathway (Marin Burgin and Szczupak 2003), the synaptic responses of T cells further support the hypothesis that the widespread action of fluoxetine was caused by excitation of an interneuronal layer that conveys signals delivered by P cells.

An interneuronal layer as a possible target of the fluoxetine effect

Besides branching in the ganglion of origin, mechanosensory neurons extend neurites to both anterior and posterior ganglia (Yau 1976). However, their signals reach neurons along the cord through interneurons that span the nerve cord (Carreta et al. 1981; Shaw and Kristan 1995; Szczupak and Kristan 1995). Unfortunately, the majority of these neurons have not yet been identified.

To test the hypothesis that the effects of fluoxetine were caused by the activation of an interneuronal layer spanning the nerve cord, we carried out a series of experiments in which we analyzed the effects of fluoxetine along chains of ganglia, using a split-bath chamber (see METHODS). This experimental configuration allowed us to expose only 2 ganglia to fluoxetine, whereas the other 3 ganglia remained in physiological saline (Fig. 7A). If the postulated hypothesis is true, then excitation of the interneurons in the ganglia exposed to fluoxetine should carry the signal to ganglia that are not exposed to the SSRI, out of the Vaseline well. In these studies we concentrated on the effect of the SSRI on the electrophysiological activity of Rz neurons. We recorded one Rz neuron in a ganglion exposed to fluoxetine, and another Rz neuron in a ganglion maintained in normal saline. It is important to bear in mind that Rz neurons of different ganglia are not connected, either electrically or chemically. As it is illustrated in Fig. 7B, we observed that both Rz neurons showed correlated synaptic activity (\( n = 3 \) preparations).

To rule out the leakage of fluoxetine to the physiological saline compartment, we cut the connectives that link adjacent ganglia outside the Vaseline well. In this condition, only the Rz neuron in the fluoxetine-exposed ganglia showed breggves of spontaneous synaptic activity, whereas the Rz neuron in the normal saline preserved its control activity.

These results support the hypothesis that the effects elicited by fluoxetine arose from the activation of a layer of interneurons that span the nerve cord. We reached this conclusion after we ruled out that fluoxetine elicited the activation of the P mechanosensory neurons, the neuronal elements in the network that were the most likely activators of the widespread synaptic effects described in Figs. 1, 4, and 5. However, it is important to consider that neurotransmitter release from mechanosensory neurons could have been enhanced without eliciting any electrophysiological activity recordable at the soma (e.g., by direct depolarization of their nerve terminals). If this alternative explanation were true, it should be possible to abolish the fluoxetine effect using an antagonist of the postsynaptic receptors present on the interneurons.

Mechanosensory P cells release glutamate and this neurotransmitter binds to non-NMDA (N-methyl-D-aspartate) type of receptors that can be blocked by the antagonist CNQX (Baccus et al. 2000; Brodfuehrer and Thorogood 2001; Wessel et al. 1999). To test whether the fluoxetine effect was exerted at or downstream of the mechanosensory output, we performed experiments (\( n = 5 \)) similar to those described in Fig. 7B, but exposing the ganglia contained in the Vaseline well to fluoxetine and CNQX. If fluoxetine caused an increase in glutamate...
release, CNQX should abolish the responses of Rz neurons inside and outside the Vaseline well. As it is illustrated in Fig. 7C, the Rz neurons in physiological saline showed the typical spontaneous synaptic activity evoked by fluoxetine, whereas the Rz neurons exposed to fluoxetine and CNQX showed little activity.
These results strongly suggest that the effects elicited by fluoxetine occurred downstream from the mechanosensory element of the network. That excitation of the interneuronal layer is the main target of the effects elicited by fluoxetine derives from the fact that Rz cells outside the Vaseline well showed the typical spontaneous barrage of EPSPs. In addition, the recordings reveal that the interneurons deliver their signal onto Rz neurons through the activation of CNQX-sensitive receptors.

Effect of serotonin on the electrophysiological activity of neurons in isolated leech ganglia

In these series of experiments we studied the effects evoked by exogenous 5-HT on the electrophysiological activity of the serotonergic Rz neurons and the AE motoneurons in single leech midbody ganglia, following a protocol similar to that shown in Fig. 1. Our goal was to compare the effects produced by exogenous 5-HT with those produced by fluoxetine.

In this series of experiments, Rz neurons (n = 13) and AE motoneurons (n = 12), studied in physiological saline, had a membrane potential of \(-53.8 \pm 2.0\) mV and \(-35.8 \pm 1.6\) mV, respectively, and showed a spontaneous firing frequency of \(0.3 \pm 0.2\) and \(3.2 \pm 0.6\) Hz, respectively. When the superfusion was switched to a solution containing 100 \(\mu\)M 5-HT, the membrane potential of both neurons shifted to a more negative value, with a concomitant decrease in spontaneous firing (Fig. 8A). By around 50 s after the onset of the 5-HT superfusion, the Rz and AE neurons were significantly hyperpolarized by \(-7.3 \pm 1.3\) mV (n = 9; P < 0.05) and \(-1.7 \pm 0.5\) mV (n = 9; P < 0.05), respectively. Because of this hyperpolarization, the spontaneous firing of the Rz neuron was abolished (n = 9; P < 0.05) and the firing frequency of the AE motoneuron decreased to \(0.3 \pm 0.2\) Hz (n = 8; P < 0.05). Note that, subtracting the delay inherent to the superfusion system (see METHODS), exogenous 5-HT produced its maximal change in membrane potential about 5 s after the full exchange of solution in the recording chamber.
To analyze whether this hyperpolarizing effect was exerted directly on the studied neurons, and to evaluate the putative ionic conductance involved, we adopted a different method to expose the neurons to the monoamine. The applications were performed by pressure pulses (1 s) from a micropipette loaded with 5-HT (1 mM in the pipette) directed toward the Rz and AE somata. The response of each neuron to 5-HT pressure pulses was studied as the neurons were shifted to different membrane potentials. These experiments were carried out in a solution containing a high Mg²⁺ membrane potentials. These experiments were carried out in a solution containing a high Mg²⁺

These results indicate that application of exogenous 5-HT evoked 2 types of effects, in different temporal windows. In the short term (seconds), it exerted a predominantly inhibitory effect on AE and Rz neurons. This effect was exerted directly on the studied neurons, as suggested by the fact that it also took place in the presence of a high Mg²⁺ concentration. In the longer term (minutes), 5-HT induced spontaneous IPSPs in AE neurons, suggesting an excitatory effect on AE inhibitory afferents.

It is worth mentioning that the concentration of 5-HT used in the present study (100 μM) was similar to that used in the previously mentioned studies in the leech (see Introduction), and was within the range (10–100 μM) of those used in other invertebrates (Marinesco and Carew 2002) and vertebrates (Andrade and Nicoll 1987; Beato and Nistri 1998). In fact, Marinesco and Carew (2002) estimated that the effective concentration of exogenous 5-HT reaching the synaptic region in Aplysia was diluted 50 times because of diffusion barriers and active serotonin-transport mechanisms.

**DISCUSSION**

**Fluoxetine as a tool to manipulate endogenous 5-HT**

The effects of fluoxetine presented in this study were interpreted under the assumption that its main action was to induce the localized accumulation of spontaneously released 5-HT from endogenous sources. The effectiveness of 5-HT uptake blockers, active in mammals, had been directly assessed in the leech nervous system (Bruns et al. 1993; Henderson 1983). In the present study, that interpretation is supported by the following experimental observations: 1) a direct effect of fluoxetine on the recorded neurons is improbable because fluoxetine had no effect when tested in a solution that inhibited chemical synaptic transmission; 2) the effect of the SSRI occurred after a delay of about 2 min, interpreted as the period required to elevate the extracellular 5-HT concentration; 3) the effect of fluoxetine was mimicked by uptake blockers of a different chemical nature, like imipramine and clomipramine, whose lower efficacy in evoking spontaneous synaptic inputs is in
agreement with their lower selectivity as 5-HT transporter blockers (Wong et al. 1995; 4) the effect of fluoxetine was mimicked by citalopram, a highly selective SSRI (Popik 1999; Stanford 1996), whose effect was at least as potent as that of fluoxetine and had a duration 3 times longer.

The analysis of the effects produced by acute blockade of 5-HT reuptake on neuronal networks has been reported in only a few previous studies. Among them, in a study of the Tritonia swimming network, 5-HT reuptake blockers were used in conjunction with stimulation of 5-HT neurons to show that 5-HT reuptake is involved in controlling the amplitude and time course of modulatory and synaptic effects by 5-HT (Katz and Frost 1995). This study reported that bath applied 5-HT can occlude both endogenous 5-HT effects, presumably because it caused activation of 5-HT receptors with opposite actions. In the respiratory system of mammals, where 5-HT plays a complex array of effects (Ballanyi et al. 1999), fluoxetine per se evoked responses similar to those generated by the activation of descending 5-HT pathways and different from those of exogenously applied 5-HT (Lalley 1986). The authors suggested that the difference resides in the targets reached by endogenous versus exogenous 5-HT. Differently, the effect of exogenous 5-HT on the swimming motor pattern of the lamprey mimicked that evoked by citalopram (Matsushima and Grillner 1992).

Effects exerted by 5-HT depend on the way by which the monoamine accesses the network

The results presented throughout this study suggest that, regarding the electrophysiological activity of certain neurons in

![FIG. 8. Rz and AE neurons hyperpolarized in response to exogenous 5-HT. A: traces show simultaneous intracellular recordings of Rz and AE neurons obtained in an isolated ganglion maintained under constant superfusion. At the beginning of the traces, the membrane potential of both neurons was about −45 mV. Bar beneath the recordings represents a superfusion timeline where the white segment indicates superfusion with physiological saline and the gray segment indicates superfusion with a solution containing 100 μM 5-HT. B: recordings of Rz and AE neurons subjected to pressure pulses of 5-HT on their somata as their membrane potential was shifted to different values (indicated on the left, in mV). Black bars below each recording series indicate the timing of the 5-HT pressure pulse (1 s in duration, serves as timescale). C: graphs show the relationship between the amplitude of the hyperpolarizations evoked by pressure pulses of 5-HT on the soma of each cell, as a function of their membrane potential (n = 4 and 5 for Rz and AE, respectively). Data in each plot were fitted to a line (r = 0.98 and r = 0.94 for Rz and AE, respectively).](https://www.jn.org/content/93/5/2652)

![FIG. 9. Delayed effects of exogenous 5-HT on AE neurons. Recordings of the same pair of neurons shown in Fig. 1A, 7 min after the onset of 5-HT (100 μM) superfusion. Fragment of AE recording enclosed in the box was expanded in the time axis and is shown beneath to allow a better observation of the dynamics of the IPSPs. Spontaneous IPSPs are indicated by the asterisks below the recordings.](https://www.jn.org/content/93/5/2652)
the leech nervous system, bath-applied 5-HT evoked radically different effects than 5-HT released from endogenous sources. Bath-applied 5-HT caused the concomitant hyperpolarization of Rz and AE neurons. The hyperpolarization was mediated by direct activation of extrasynaptic 5-HT receptors, as suggested by the fact that pulses of 5-HT ejected onto the somata of Rz and AE neurons evoked the same responses as those of bath application. In contrast, raising extracellular 5-HT from endogenous sources, as a result of inhibiting its reuptake, caused an excitatory phenomenon that resulted in the activation of coincident synaptic inputs to a variety of neurons. Thus the responses recorded in the different neurons that have been investigated were not the outcome of a direct interaction between the endogenously released 5-HT and the recorded neurons, but were attributed to the activation of presynaptic interneurons. In other words, we interpret that the increase in extracellular 5-HT concentration, close to its release sites, primarily affected interneurons presynaptic to the recorded neurons. Figure 10 presents a schematic representation of this interpretation.

We consider that these distinct actions of 5-HT, depending on the method of its delivery to the network, were attributable to the activation of different 5-HT receptors that exhibit a differential spatial distribution (Kristan and Nusbaum 1983). Bath-applied 5-HT (generating a homogeneous 5-HT concentration through all the ganglion) probably activated extrasynaptic 5-HT receptors, widely distributed on the surface of the recorded neurons, including the somata (Sargent et al. 1977). In contrast, 5-HT accumulated from endogenous pools (generating a 5-HT concentration gradient from the release sites) activated receptors that probably exhibit a more restricted distribution.

Our work shows that serotonergic neuromodulation in the leech follows similar principles as those illustrated in the studies performed in Tritonia swimming network and in the mammalian respiratory network, described in the previous section, in that the monoamine access to the network is physiologically relevant. Here we show clear evidences that 5-HT released from endogenous sources selectively reached only part of its putative targets, causing a specific network outcome. A similar “spatial interpretation” was proposed in the stomatogastric network of the crab regarding endogenous versus bath-applied proctolin (Blitz and Nusbaum 1999; Wood and Nusbaum 2002).

The S interneuron represents a special case within this study. In a previous work that analyzed the effects of 5-HT on the S interneurons (Burrell et al. 2001), it was found that bath-applied 5-HT increases the excitability of the S cells. In our study, fluoxetine mimicked the excitatory effect of 5-HT on the S cell, as revealed by an increase in its basal tonic firing frequency (Fig. 5). Thus regarding S cells, exogenous and endogenous 5-HT acted similarly, suggesting that the 5-HT release sites could be close to the region of the S cell bearing the 5-HT receptors that affect its excitability. In addition, the S cells were also a target of the interneuronal layer (Fig. 10B).

The neuronal target of endogenous 5-HT

Based on the assumption that the fluoxetine effects observed in the present study arose from its SSRI activity, our results lead to 3 main conclusions: 1) there is a constant basal release of 5-HT in the leech ganglion that is usually restricted by reuptake mechanisms; 2) the main target of this 5-HT source is a layer of interneurons with widespread action on several motoneurons and interneurons and on T sensory neurons (Fig. 10B); and 3) this interneuronal layer spans several ganglia (Fig. 10A).

The existence of a basal 5-HT release in the leech was already suggested by Willard (1981), who showed that there was a correlation between the concentration of 5-HT in the blood and the level of spontaneous motor activity displayed by individual leeches. However, it remains to be investigated whether the endogenous source of 5-HT that supplies the blood is the same as that affected by the acute application of fluoxetine analyzed in this study. The results also suggest that the effects of 5-HT present in the circulation may be opposed to that caused by a more focal release and the overall effect is probably derived from the balance between the effects evoked by these 5-HT sources.

The existence of an interneuronal layer spanning the nerve cord that conveys sensory signal to all segments was already documented in the leech (Shaw and Kristan 1995; Szczupak and Kristan 1995). We consider that the increased excitability displayed by the S cells in the presence of 5-HT constitutes a
possible explanation of how the endogenously released 5-HT could have affected the interneuronal layer. Thus the wide synaptic activity elicited by endogenous 5-HT could derive, at least in part, from a decrease in the firing threshold of the neurons constituting the interneuronal layer. As a result, spontaneous ganglionic activity, which in normal conditions would cause subthreshold responses in the cord-spanning interneurons, could turn suprathreshold when the extracellular 5-HT was increased. However, it is important to note that the S cell does not have the connectivity profile to explain the results described in Figs. 1, 4, and 6.

The physiological implication of these results is that, although the serotonergic system has a wide range of actions, because of the presence of numerous receptors located in a broad variety of neurons, its pattern of action is substantially determined by the spatial distribution of the 5-HT release sites. The location of these sites would grant an adequate 5-HT gradient that hits the “right” receptors in the “right” order. This agrees with the view that 5-HT acts within neuromodulatory fields restricted to regions close to its release sites (Marinesco and Carew 2002). In this context, our observations suggest that the 5-HT release sites affected by fluoxetine in the leech nervous system are targeted toward interneurons with wide-spread synaptic actions.

REFERENCES


