SELECTIVE SEROTONIN REUPTAKE INHIBITORS INDUCE
SPONTANEOUS INTERNEURONAL ACTIVITY IN THE LEECH NERVOUS
SYSTEM.

Running head: Effects of SSRIs on the leech nervous system

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ABSTRACT

Serotonin (5-HT) is a conspicuous neuromodulator of sensory-motor networks that affects a variety of neurons at different levels of the network hierarchy. Due to its many possible targets, it has been difficult to obtain a comprehensive picture of how 5-HT achieves its final modulatory output on any given network. Our hypothesis is that the profile of 5-HT actions is dictated by its pattern of release from endogenous sites. We tested this hypothesis in the leech nervous system by means of a selective serotonin reuptake blocker (SSRI), fluoxetine. Fluoxetine evoked barrages of synaptic potentials in identified sensory, motor and inter-neurons. 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 via polysynaptic pathways. The results suggest that endogenous 5-HT acted upon cord spanning interneurons. On the other hand, bath-applied 5-HT evoked a different effect than the SSRI. Taken together, the results evidenced that the pattern of action of the monoamine is dictated by the spatial distribution of the 5-HT release sites.

Keywords: leech – serotonin – interneurons – selective serotonin reuptake inhibitor - neuromodulation
INTRODUCTION

Serotonin (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; Schmidt and Jordan 2000; Jankowska 2001).

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 5HT receptors (Barnes and Sharp 1999). Given the striking parallel roles of 5-HT in vertebrates and invertebrates, the analysis of serotonergic systems in the invertebrates poses advantages for understanding functional principles (Jacobs and Fornal 1993). Because of the relative simplicity of certain invertebrates, it is possible to study the 5-HT effects in a more integrative mode while retaining a cellular approach (Marder 2002).

The long-term objective of this investigation is to understand the role of endogenous 5-HT in sensory-motor networks, 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 inter-neurons can be recorded simultaneously.

In the leech, serotonin increases the probability of producing the swim motor pattern (Willard 1981; Hashemzadeh-Gargari and Friesen 1989). This monoamine altered the physiological properties of swim-initiating neurons (Angstadt and Friesen 1993a; Angstadt and Friesen 1993b), motoneurons (Mangan et al. 1994a; Mangan et al. 1994b) and muscle fibres (Mason and Kristan 1982), favoring the induction of swimming. It has been suggested that 5-HT induces swimming, acting via paracrine and synaptic effects
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), as in other organisms (Andrade and Nicoll 1987; Teshiba et al. 2001; Barbas et al. 2003), 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 inter-neurons and compare them with those evoked by bath-applied 5-HT. Based on the effectiveness of 5-HT reuptake inhibitors in the leech (Henderson 1983; Bruns et al. 1993), 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 than those evoked by bath-applied 5-HT.
MATERIALS AND METHODS

Biological preparation

*Hirudo medicinalis*, weighing 2-5 g, were obtained from a commercial supplier (Leeches USA, Westbury, NY) and maintained at 15°C in artificial pond water. The animals were not fed for at least one month prior to 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 undistinguishable from one another. Each one bears a full set of sensory and motor neurons, while the interneurons show three different configurations: a) neurons that are confined within each ganglion; b) neurons that have their somata in one ganglion and extend arborizations toward several anterior or posterior ganglia; or c) descending interneurons from either of the two 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 Figure 7, the preparation consisted of a chain of five ganglia in a split-bath chamber. In this recording configuration, two ganglia of the chain were surrounded by a Vaseline™ well to form a waterproof seal that separated them chemically from the other three ganglia. The solution in the Vaseline well was
under constant superfusion, while 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 composition 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 mm x 250; 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 pmoles/ganglion, n = 2) during the fluoxetine-insensitive period, than during periods of normal activity (26.72 ± 5.82 pmoles/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 and 1 mM CaCl₂), in which the osmolarity was kept constant by reducing the NaCl concentration. We choose a concentration of 7 mM Mg²⁺, rather than the classically used 20 mM (Baylor and Nicholls 1969), in order to preserve the excitability of the neurons. To test the effectiveness of different Mg²⁺
concentrations, we studied the interaction between the mechanosensory P cell and the AE motoneuron (Iscla et al. 1999). In the presence of 7 mM Mg$^{2+}$/1 mM Ca$^{2+}$, the P-AE interaction was completely and reversibly abolished ($n = 5$), but both neurons retained their normal excitability upon direct electrical stimulation.

The different drugs used in this study were dissolved in physiological saline and applied through the 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), fluoxetine, imipramine and citalopram mesylate were purchased from Sigma-Aldrich Co. (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 ± SEM (standard error of the mean) and the number of independent observations is expressed between brackets (n). Statistical significance of the results obtained in different experimental conditions was determined by Student t-test. Curve fitting was achieved using a commercial software (Kaleidagraph 3.0.2, Abelbeck Software, Reading, PA)

In order 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 Rz recordings, using a bin size of 12.5 msec.
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 (shortly described in Materials and 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 have been initially chosen to monitor the activity evoked by fluoxetine: the AE motoneuron, responsible for erection of the annuli that subdivide the leech skin (Stuart 1970; Rodriguez et al. 2004), 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 two types of neurons do not bear any kind of synaptic interactions among 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), while the AE neuron displays inhibitory synaptic potentials (IPSPs). While in control conditions Rz neurons fired at a steady frequency, the beginning of the fluoxetine effect was marked by small bursts of, at least, two to three 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, while 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 Material and 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, in order 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 \( \frac{Mg^{2+}}{Ca^{2+}} \) ratio that prevents neurotransmitter release in the leech (see Materials and 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 Figure 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 duration, they had a similar onset. To evaluate the degree of coincidence between the responses of Rz and AE neurons, a cross-correlation analysis (see Materials and Methods) was implemented using recording segments (60 sec) 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 ($n = 5$), the Rz neurons presented EPSPs of low amplitude and the action potential bursts were composed of a few spikes ($n = 9$), or the responses of both cells were very low ($n = 10$). All these cases ($n = 24$) 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 due to 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 vs. 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 (Stanford 1996; Popik 1999).

The experiments followed a similar protocol to that described in Figure 1. Clomipramine ($n = 7$ out of 7) or imipramine ($n = 4$ out of 8) evoked spontaneous synaptic events in Rz and AE neurons that were qualitatively similar to those evoked by fluoxetine, but 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 $50 \mu M (n = 3)$ and $100 \mu M (n = 3)$ 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 a Rz and an AE neuron evidencing a highly correlated occurrence of EPSPs and IPSPs. The synaptic events induced by citalopram ($100 \mu M$) started $1.67 \pm 0.33$ min after the superfusion was initiated (an actual delay of 57 sec), and lasted for at least $13.33 \pm 1.33$ 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 due 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 (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 non-spiking 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 on 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 elicted 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 two main targets: a) the mechanosensory P neurons or, b) 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 three 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 three pairs of T cells and two pairs of N and P cells.

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

Since T cells receive inhibitory signals from P and N cells through a polysynaptic pathway (Marín 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; Szczupak and Kristan 1995; Shaw and Kristan 1995). Unfortunately, the majority of these neurons have not been identified yet.

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 Materials and Methods). This experimental configuration allowed us to expose only two ganglia to fluoxetine, while the other three 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 neither electrically nor chemically. As it is illustrated in Figure 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 barrages of spontaneous synaptic activity, while the Rz neuron in the normal saline preserved its control activity. These results support the hypothesis that the effects elicited by fluoxetine were due to the activation of a layer of interneurons that span the nerve cord. We reached to 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 Figures 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 was 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 type of receptors that can be blocked by the antagonist CNQX (Wessel et al. 1999; Baccus et al. 2000; Brodfuehrer and Thorogood 2001). 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 Figure 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 Figure 7C, the Rz neurons in physiological saline showed the typical spontaneous synaptic activity evoked by fluoxetine, while the Rz neurons exposed to fluoxetine and CNQX showed little activity.

These results strongly suggest that the effects elicited by fluoxetine took place 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 similar protocol as that shown in Figure 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 \(-53.8 \pm 2.0\) and \(-35.8 \pm 1.6\) mV, respectively, and showed a spontaneous firing frequency of \(0.3 \pm 0.1\) 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 ± 1.3 mV (n = 9; P < 0.05) and –1.7 ± 0.5 mV (n = 9; P < 0.05), respectively. Due to this hyperpolarization, the spontaneous firing of the Rz neuron was abolished (n = 9; P < 0.05) and the firing frequency of the AE motoneuron dropped to 0.3 ± 0.2 Hz (n = 8; P < 0.05). Note that, subtracting the delay inherent to the superfusion system (see Material and Methods), exogenous 5-HT produced its maximal change in membrane potential about 5 sec 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 sec) 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⁺/Ca²⁺ ratio to isolate the 5-HT effect on these neurons from putative effects through other neurons via chemically-mediated synapses. Figure 8B shows representative examples of the responses of a Rz neuron and an AE motoneuron that illustrate that pressure pulses of 5-HT evoked transient hyperpolarizing responses whose amplitude decreased as the membrane potential of the neurons was shifted to negative values. The plots in Figure 8C summarize the results obtained from this series of experiments. The linear regressions in the graphs indicate that the responses of Rz and AE reversed at around -70 mV, suggesting that they were produced by the activation of a chloride conductance (Walker and Smith 1973).
This observation agrees with previous studies showing that exogenous 5-HT causes the activation of a chloride current in Rz neurons (Walker and Smith 1973; Munsch and Schlue 1993) and in other neurons (Henderson 1983; Sanchez-Armass et al. 1991).

In addition to the short latency effect, superfusion with 5-HT evoked a long latency effect in the AE motoneurons. While Rz neurons remained silent throughout the exposure to the 5-HT solution (100 µM), approximately half of the AE neurons showed a delayed discharge of spontaneous inhibitory postsynaptic potentials (IPSPs). Figure 9 shows representative recordings exhibiting these phenomena. The IPSPs appeared around 6 min after the onset of the 5-HT superfusion (6.0 ± 0.8 min, \( n = 4 \) out of 7 ganglia). Noticeably, a concentration of 1 µM 5-HT sometimes evoked the long-latency effects (\( n = 2 \) out of 4 ganglia) although it did not evoke any change in the resting potential of the AE cells (\( n = 4 \), data not shown).

These results indicate that application of exogenous 5-HT evoked two types of effects, in different temporal windows. In the short-term (sec), 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 \( \text{Mg}^{2+} \) concentration. In the longer term (min), 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 (Henderson 1983; Bruns et al. 1993). 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 since fluoxetine had no effect when tested in a solution that inhibited chemical synaptic transmission; 2) the effect of the SSRI took place 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 (Stanford 1996; Popik 1999), whose effect was at least as potent as that of fluoxetine and had a duration three 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 than those of exogenously applied 5-HT (Lalley 1986). The authors suggested that the difference resides in the targets reached by endogenous vs. 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).

**The 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 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 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 due to the activation of presynaptic interneurons. In other
words, we interpret that the increase in extracellular 5-HT concentration, close to its release sites, affected primarily 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 due to the activation of different 5-HT receptors that exhibit a differential spatial distribution (Kristan and Nusbaum 1983). Bath-applied 5-HT (generating an 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 vs. 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 were
due to its SSRI activity, our results lead to three 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 studied 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 two 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 (Szczupak and
Kristan 1995; Shaw 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 comprising the interneuronal layer. As a result, spontaneous ganglionic activity, that 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 Figures 1, 4 and 6.

The physiological implication of these results is that although the serotonergic system has a wide range of actions, due to the presence of numerous receptors located in a broad variety of neurons, its pattern of action is highly 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 widespread synaptic actions.
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LEGENDS TO FIGURES

Figure 1. Fluoxetine caused spontaneous synaptic activity in Rz and AE neurons.
The traces show simultaneous intracellular recordings of a Rz and an AE neuron obtained in an isolated ganglion maintained under constant superfusion. The lower pair of traces is the continuation of the upper pair. At the beginning of the upper traces, the membrane potentials of the Rz and the AE neurons were about -45 and -35 mV, respectively. The bar underneath each pair of recordings represents a superfusion-time-line where the white segment indicates superfusion with physiological saline and the gray segment indicates superfusion with a solution containing 100 µM fluoxetine. The asterisk denotes the onset of the fluoxetine effect, as defined in the Results. Note that at the time scale displayed in this figure, the action potentials of the AE neurons are not distinguishable individually and they form the apparent thick line. The recording fragments enclosed in the box was expanded in the time axes and shown in the inset underneath. At this time-scale the spikes and IPSPs recorded in the AE neuron can be individually distinguished.

Figure 2. The synaptic responses of Rz and AE neurons were temporally correlated.
The graph shows the average cross-correrogram \((n = 14)\) between AE and Rz recordings. The cross correlation analysis was performed using fragments of 60 sec out of the period the maximal activity evoked by fluoxetine (see Materials and Methods). The dotted lines represent the confidence range \((\pm 2 \text{ SEM})\) for the mean cross-correlation index.

Figure 3. Spontaneous synaptic activity in Rz and AE neurons exposed to different 5-HT reuptake inhibitor.
A. Representative responses of a Rz and an AE neuron to superfusion with a solution containing imipramine \((100 \mu M)\). The fragment enclosed in the box is shown underneath
in an expanded time scale. **B.** Representative responses of Rz and AE recordings to superfusion with a solution containing citalopram (100 µM).

**Figure 4. Responses of different identified neurons in the leech midbody ganglion to fluoxetine.**

The traces show fragments of simultaneous paired recordings of neurons in isolated ganglia in normal saline (CONTROL) and during the superfusion with 100 µM fluoxetine. The paired recordings correspond to a Rz neuron and the following neurons:

A) CV, B) AP, C) NS, D) cell 204, and E) cell 61. The membrane potentials (in mV) at the baseline of each of the recordings (Rz & the second cell, respectively) are: in control: A) –35 & –38; B) –57 & –55; C) –55 & –40; D) –55 & –42; and E) –30 & –30. In Fluoxetine: A) –30 & –40; B) –45 & –50; C) –50 & –45; D) –50 & –50; and E) –28 & –30.

**Figure 5. Dual effect of fluoxetine on the S interneuron.**

The traces show simultaneous intracellular recordings of a Rz and an S neuron obtained in an isolated ganglion maintained under constant superfusion. At the beginning of the traces the membrane potentials of the Rz and the S neurons were about -40 and -35 mV, respectively. The bar underneath the recordings represents a superfusion-time-line where the white segment indicates superfusion with physiological saline and the gray segment indicates superfusion with a solution containing 100 µM fluoxetine. The recording fragment enclosed in the box was expanded in the amplitude and time axes and shown underneath.

**Figure 6. Responses of mechanosensory neurons and Rz neurons to superfusion**
with fluoxetine.

A. Simultaneous recordings of a Rz neuron and a mechanosensory P neuron obtained from an isolated ganglion during superfusion with a solution containing fluoxetine (100 \( \mu \text{M} \)). The baseline membrane potential for Rz was about –50 mV and for P –35 mV. B. Simultaneous recordings of a Rz neuron and a mechanosensory T neuron obtained from an isolated ganglion during fluoxetine superfusion (100 \( \mu \text{M} \)). The baseline membrane potential for Rz was about –60 mV and for T –45 mV.

Figure 7. The effect of fluoxetine was transmitted to Rz neurons along the nerve cord.

A. The diagram describes the recording configuration used in the experiment: a chain of five interconnected midbody ganglia (represented by large circles) from MG7 to MG11 were mounted in a split-bath chamber (see Materials and Methods) and the Rz neurons (represented as pairs of little circles in the center of each ganglion) were recorded. The gray box represents the Vaseline well where fluoxetine was perfused in, that enclosed MG10-MG11. Notation: Rz (7), Rz (8) and Rz (11) correspond to Rz neurons in MG7, MG8 and MG11, respectively. B. The traces show simultaneous recordings of Rz (8) and Rz (11) as the extracellular saline contained in the Vaseline well was switched from physiological to one containing 100 \( \mu \text{M} \) fluoxetine (the switch took place at the beginning of the traces). The baseline membrane potential of both Rz cells was about –60 mV. C. The traces are simultaneous recordings of Rz (11) and Rz (7) during superfusion with a solution containing fluoxetine (100 \( \mu \text{M} \)) and CNQX (100 \( \mu \text{M} \)) in the Vaseline well. The baseline membrane potential of both Rz cells was about –50 mV.
Figure 8. Rz and AE neurons hyperpolarized in response to exogenous 5-HT.

A. The traces show simultaneous intracellular recordings of a Rz and an AE neuron 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. The bar underneath the recordings represents a superfusion-time-line 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 a Rz and an AE neuron 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). The black bars underneath each recording series indicate the timing of the 5-HT pressure pulse (1 s in duration, serves as time scale). C. The 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). The data in each plot was fitted to a line (r = 0.98 and r = 0.94 for Rz and AE, respectively).

Figure 9. Delayed effects of exogenous 5-HT on AE neurons.

Recordings of the same pair of neurons shown in Figure 1A, seven minutes after the onset of 5-HT (100 µM) superfusion. The fragment of AE recording enclosed in the box was expanded in the time axis and shown underneath to allow a better observation of the dynamics of the IPSPs. The spontaneous IPSPs are indicated by the asterisks underneath the recordings.

Figure 10. Diagram of the neuronal interactions.

A. Diagram of a chain of five leech midbody ganglia in which we line out the neuronal
interactions that underlie the experimental results as interpreted in the text. We assume similar connectivity in each ganglion. B. Expanded view of the connectivity in a single ganglion: an interneuronal layer (represented by the thick gray line) makes excitatory or inhibitory synaptic connections with sensory (T), effector (Rz, AE, AP and CV), premotor (NS) and inter- (204 and S) neurons (represented as white circles). The interneuronal layer receives excitatory input from the sensory P cell (black circle). The gray surface indicates the effective spatial extent of the accumulated 5-HT, released from endogenous sources. GLU indicates glutamatergic synaptic transmission. According to our interpretation the accumulated 5-HT affects primarily the interneuronal layer (and the S cell) and, through it the effector, sensory and interneurons.
sensory interneurons effector

T
P

interneuronal layer

GLU glutamatergic synapse

endogenous 5-HT

GLU excitatory synapse

GLU inhibitory synapse

A

B

sensory interneurons effector

T
P

GLU

Rz
AE
AP
CV
NS
204
S