MULTIPLE RECEPTORS MEDIATE THE MODULATORY EFFECTS OF SEROTONERGIC NEURONS IN A SMALL NEURAL NETWORK

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Summary

The gastropyloric receptor (GPR) cells are a set of cholinergic/serotonergic mechanosensory neurons that modulate the activity of neural networks in the crab stomatogastric ganglion (STG). Stimulation of these cells evokes a variety of slow modulatory responses in different STG neurons that are mimicked by exogenously applied serotonin (5-HT); these responses include tonic inhibition, tonic excitation and induction of rhythmic bursting. We used pharmacological agonists and antagonists to show that these three classes of modulatory response in the STG neurons are mediated by distinct 5-HT receptor subtypes. GPR stimulation or application of 5-HT or 2-me-5HT (a vertebrate 5-HT3 agonist) inhibited the pyloric constrictor (PY) neurons; these actions were selectively antagonized by gramine. GPR stimulation or application of 5-HT induced rhythmic bursting in the electrically coupled anterior burster (AB) and pyloric dilator (PD) neurons; these effects were antagonized by the 5-HT1c/2 antagonist cinanserin and by atropine at concentrations that do not block muscarinic cholinergic receptors in the crab STG. The 5-HT agonists 5-CT (5-HT1) and a-me-5HT (5-HT2) also induced AB/PD bursting, which was blocked by cinanserin, but not by atropine. GPR stimulation or application of 5-HT and 5-CT evoked tonic excitation of the lateral pyloric (LP) neuron. These effects were blocked by cinanserin. Several other 5-HT agonists and nearly all the vertebrate 5-HT antagonists we tested had little or no effect on the crab pyloric 5-HT receptors. These results provide further evidence that the modulatory sensory GPR neuron uses serotonin to evoke multiple modulatory responses via multiple 5-HT receptors. However, the 5-HT receptors in the crab STG neurons are not pharmacologically similar to vertebrate 5-HT receptors.

Introduction

Serotonin, or 5-hydroxytryptamine (5-HT), is known to be a neurotransmitter in both vertebrate and invertebrate nervous systems (Amin et al. 1954; Brodie and Shore, 1957;
Welsh and Moorehead, 1960; Maynert et al. 1964; Gerschenfeld and Paupardin-Tritsch, 1974a,b; Beltz et al. 1984; Katz et al. 1989; Katz and Harris-Warrick, 1989, 1990a). Our knowledge of the mechanisms controlling the action of 5-HT has been enhanced through studies of its receptor types using selective ligands, especially in the vertebrates. Since Gaddum and Picarelli (1957) first proposed two kinds of 5-HT receptor, studies in vertebrate tissues have demonstrated at least four major 5-HT receptor types and a number of subtypes (Bradley et al. 1986; Schmidt and Peroutka, 1989; Andrade and Chaput, 1991a). It is now generally believed in vertebrates that different excitatory or inhibitory responses are mediated by distinct 5-HT receptors (Aghajanian, 1981; Bobker and Williams, 1990; Andrade and Chaput, 1991b). However, little is known about 5-HT receptor types in the nervous systems of invertebrates.

We have been studying the physiological roles of serotonin in the crab stomatogastric ganglion (STG). The STG contains about 30 neurons comprising two central pattern generator (CPG) networks controlling the rhythmic movements of crab foregut muscles (Selverston and Moulins, 1987). A set of four peripheral mechanosensory neurons, called the gastropyloric receptor (GPR) cells, is activated by tension at the gastropyloric border of the foregut. The GPR cells contain both 5-HT and choline acetyltransferase, the synthetic enzyme for acetylcholine (ACh). It has been proposed that the GPR cells use both 5-HT and ACh as co-transmitters to modulate the motor patterns generated by the STG (Katz et al. 1989; Katz and Harris-Warrick, 1989, 1990a, 1991). The traditional role of mechanosensory cells is to provide rapid phasic feedback to the motor network. The GPRs accomplish this by releasing ACh, which evokes fast nicotinic cholinergic excitatory postsynaptic potentials in a variety of postsynaptic neurons. A new neuromodulatory role for mechanosensory cells was observed when the GPRs induced prolonged modulatory changes in the motor patterns generated by the crab CPGs (Katz and Harris-Warrick, 1989, 1990a). These modulatory effects are mimicked by exogenous 5-HT and vary among the different STG neurons. Some neurons respond to GPR or 5-HT with tonic inhibition; others exhibit tonic excitation, rhythmic bursting or plateau potentials.

These different cellular responses to 5-HT could be explained by two general classes of mechanisms. First, the different target neurons could possess different 5-HT receptors that evoke different electrophysiological responses. Second, the neurons could share a common 5-HT receptor, with the signal being interpreted differently by the different target neurons because of differences in intrinsic cellular response properties, G protein subtypes or coupled second-messenger mechanisms. These two mechanisms are not mutually exclusive, and further permutations are possible. In this paper, we present evidence from electrophysiological and pharmacological studies that crab STG neurons possess multiple receptors recognized by 5-HT. These 5-HT receptors can be classified into several distinguishable subtypes in different pyloric neurons; each is characterized by selective agonists and antagonists. Neurons that have different physiological responses to GPR stimulation and to application of 5-HT or its agonists are sensitive to different 5-HT antagonists. These results also provide further support for our hypothesis that 5-HT is a transmitter used by the GPR cells.

Part of this work has appeared in abstract form (Zhang and Harris-Warrick, 1991).
Materials and methods

Animals

Crabs (*Cancer borealis*) were purchased from suppliers in Boston, Massachusetts. The crabs were maintained in Instant Ocean aquaria at 12–14 °C.

Saline and chemicals

The crab saline contained (mmol l\(^{-1}\)): 440 NaCl, 11 KCl, 13 CaCl₂, 26 MgCl₂, 8 glucose, 11 Trizma base buffer, and 5 maleic acid. The pH was 7.4. The following drugs were tested: atropine sulfate, cyproheptadine hydrochloride and gramine [3-(dimethylaminomethyl) indole] (purchased from Sigma); MDL 72222 (3-tropanyl-3,5-dichlorobenzoate), pirenperone, mCPP [1-(3-chlorophenyl)piperazine dihydrochloride], 5-CT (5-carboxamidotryptamine maleate), CGS-12066B dimaleate, α-methyl-5HT [(±)-α-methyl-5HT maleate], 2-me-5HT [(±)-2-methyl-5-HT maleate] and 1-phenylbiguanide (purchased from Research Biochemicals Inc.) The following drugs were generous gifts from the pharmaceutical companies indicated in parentheses: cinanserin hydrochloride (The Squibb Institute for Medical Research); metergoline (Farmitalia Carloerba); metoclopramide hydrochloride (A. H. Robins Co.); methysergide maleate (Sandoz Pharmaceuticals); mianserin hydrochloride (Organon Inc.); and spiperone (Janssen Pharmaceutica Inc.). All drug solutions were prepared immediately before use. Atropine, cinanserin and all agonists were dissolved directly into the saline. Gramine, MDL 72222, mianserin and metoclopramide were dissolved in ethanol at 10 mmol l\(^{-1}\). The other drugs were dissolved in either methanol or dimethyl sulfoxide (DMSO) at 10 mmol l\(^{-1}\). The final concentration was obtained by diluting each stock into saline. Ethanol, DMSO and methanol had no effects of their own at the final dilutions used (maximum 0.2 %). 5-HT (serotonin creatinine sulfate, Sigma) was applied by pressure ejection from an adjacent micropipette at 1 μmol l\(^{-1}\) to 1 mmol l\(^{-1}\) (see below).

Physiology

The dissection of the stomatogastric nervous system followed the methods described by Selverston *et al.* (1976). The dissection was usually performed on the day of the experiment. However, some dissections were performed up to the desheathing of the STG the previous evening; this did not affect the results in any way.

Physiological recordings were performed as described by Katz *et al.* (1989; Katz and Harris-Warrick, 1989, 1990a). Extracellular recordings were made from identified motor nerves with bipolar stainless-steel pin electrodes. Intracellular recordings were made with glass microelectrodes (15–25 MΩ) filled with 4 mol l\(^{-1}\) potassium acetate plus 0.3 mol l\(^{-1}\) potassium chloride, or 3 mol l\(^{-1}\) potassium chloride. STG neurons were identified by (1) matching action potentials recorded from the appropriate motor nerve root and intracellularly from the soma, (2) the characteristic shape of membrane potential oscillations and spike amplitudes, and (3) the timing of spike activity within the pyloric rhythm. Following neural identification, input from the anterior ganglia was blocked by filling a Vaseline ring on the desheathed stomatogastric nerve (stn) with isotonic sucrose solution in 50 mmol l\(^{-1}\) Tris–maleate buffer. Sometimes, a Vaseline chamber
surrounding the anterior ganglia was filled with the isotonic sucrose solution or saline containing 1 μmol l⁻¹ tetrodotoxin (TTX), and the stn was then desheathed.

The STG (bath volume 0.2–0.5 ml) was superfused with saline at 2 ml min⁻¹. The 5-HT agonists were bath-applied for various times (2–5 min) before the results were recorded and washed for 30–50 min afterwards. Serotonin antagonists were allowed to superfuse the ganglion for at least 15 min to ensure equilibration before any recordings were made and washed for at least 30 min between drug applications. All the drug effects reversed upon removal of the drug. Unless noted otherwise, all experiments were performed in saline containing picrotoxin (PTX, Sigma, 5 μmol l⁻¹) to eliminate the glutamate synapses between neurons (Bidaut, 1980; Eisen and Marder, 1982; Marder and Eisen, 1984; Marder, 1987). This reduced the synaptic interactions between the STG neurons, facilitating the measurement of direct cellular responses to GPR stimulation or 5-HT (Fig. 1; Katz and Harris-Warrick, 1989, 1990a).

Pressure ejection was used to ‘puff’ small volumes of 5-HT solution onto the neuropil of the STG. The puffer micropipettes had tip diameters of about 2 μm and were located approximately 80 μm downstream from the STG. Calibrated pressure pulses

Fig. 1. The pyloric circuit, adapted from Katz and Harris-Warrick (1990a). Inhibitory chemical synapses are denoted by circles. The filled circles are picrotoxin-sensitive glutamate synapses, while the open circles are cholinergic synapses. Electrotonic junctions are presented as resistors. Serotonergic inputs from the gastropyloric receptor (GPR) cells to stomatogastric ganglion neurons are denoted by triangles and bars. The triangles represent excitatory synapses, while the bars are inhibitory. Serotonergic agonists and antagonists that act at different GPR synapses are indicated.

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(300–3000 ms; 35–138 kPa) ejected the 5-HT solution over the whole ganglion. Fast Green FCF (1 mg ml\(^{-1}\), Sigma) was used as an indicator for the 5-HT ejection; the dye alone had no effect on the STG.

The identification and stimulation of the GPR cells followed the methods described by Katz et al. (1989).

Data analysis

Data presented here were collected from experiments conducted with sixty animals. Each drug was tested at least five times. Values are expressed as mean ± S.E.M. A paired Student’s t-test was used for data analysis. The difference was considered significant when the P<0.05.

Results

Katz and Harris-Warrick (1989, 1990) identified four major classes of response to GPR stimulation among neurons of the pyloric network (Fig. 1). These include transient inhibition of PY, the induction or enhancement of rhythmic bursting in the AB/PD neurons, tonic excitation of LP and biphasic responses (inhibition followed by excitation) in the inferior cardiac neuron (IC). Bath application of 5-HT mimicked the GPR response in several of these cases, although the time course of the response was very different because of the prolonged application of the amine. We have studied 5-HT receptor pharmacology in the first three classes of responses (PY, AB/PD and LP). Here we show that a brief (300–3000 ms) application of 5-HT by pressure ejection, or ‘puffing’, can more closely approximate the time course of the modulatory component of GPR stimulation. Bath application of different 5-HT agonists mimics the modulatory effects of GPR stimulation and 5-HT application in different neurons. Further, the effects of GPR stimulation and application of 5-HT or its agonists are selectively blocked by selective 5-HT antagonists in different STG neurons.

5-HT and 2-me-5HT mimic the effects of GPR in the PY neuron

GPR stimulation and a brief ‘puff’ of 5-HT (0.1 mmol l\(^{-1}\), 1–3 s) both induced a transient inhibition of bursting in the PY neuron (Katz and Harris-Warrick, 1989, 1990; Fig. 2). Note that during this inhibition the PY oscillations were selectively reduced in their peak amplitude and slowed in their rate of post-inhibitory rebound; the trough of the burst (due to synaptic inhibition from the AB/PD neurons, see Fig. 1) was unaffected. Since PY receives glutamatergic inhibition from LP, the GPR-and 5-HT-evoked transient inhibition could result from an indirect effect from the excited LP. To eliminate this possibility, we perfused the ganglion with PTX-containing saline, which blocks the synaptic inhibition from LP (Fig. 1). Under this condition, we still observed an inhibition of PY by GPR stimulation and application of 5-HT (Fig. 2B), confirming a direct effect of GPR and 5-HT on PY (Katz and Harris-Warrick, 1989, 1990).

This inhibition of the PY cell was selectively mimicked by bath application of the 5-HT\(_3\) agonist 2-me-5HT (Fig. 3). The number of spikes per PY burst was reduced (similar to the effect of bath-applied 5-HT; Katz and Harris-Warrick, 1989, 1990), and the peak
amplitude of the PY oscillation was reduced, with little change in the synaptically evoked trough of the burst. Table 1 shows that 2-me-5HT significantly reduced the number of action potentials per PY burst in a dose-dependent manner, essentially eliminating PY firing at 10 μmol l⁻¹. A number of other 5-HT agonists were tested, but only 2-me-5HT affected the PY neurons. 2-me-5HT had no effect on other pyloric neurons (see Table 4).

**Gramine blocks GPR, 5-HT and 2-me-5HT inhibition of the PY neuron**

Gramine (1–100 μmol l⁻¹) blocked the GPR-evoked inhibition of the PY neuron.
Fig. 2. Similarly, gramine blocked the inhibition evoked by a brief puff of 5-HT (0.1 mmol l\(^{-1}\); Fig. 2) and reduced the inhibition evoked by bath application of the agonist 2-me-5HT (Fig. 3). The effects of gramine completely reversed upon washout. Gramine alone had little or no effect on existing PY activity. Various other antagonists were also tested: they had no antagonistic effects on either 5-HT- or GPR-induced inhibition of PY (see Table 5). Gramine had no effect on 5-HT or GPR-evoked responses in other neurons.

5-HT, 5-CT and \(\alpha\)-me-5HT mimic the effects of GPR in the AB/PD neurons

GPR stimulation and 5-HT application also induce or enhance rhythmic bursting in the electrically coupled AB/PD neurons (Katz and Harris-Warrick, 1989, 1990a). After a sucrose block has been placed on the desheathed stn to block modulatory inputs to the STG from the anterior ganglia, the electrically coupled conditional burster PD and AB
neurons usually slow down or cease their rhythmic bursting, as do all the STG neurons (Russell, 1979). As shown in Fig. 4, a brief puff of 5-HT (1 mmol l\(^{-1}\)) or GPR stimulation (20 Hz) induces rhythmic bursting in the AB neuron. The induction of bursting in AB by both 5-HT and GPR disappears when atropine (0.1 mmol l\(^{-1}\)) is added to the control saline (B). The resting potential for the AB neuron is \(-63\) mV, which remains unchanged in the presence of atropine. Note that an initial hyperpolarization of the AB typically follows the puff of 5-HT. This hyperpolarization is caused in part by IPSPs (arrows) from simultaneous excitation of the LP neuron (not shown). Atropine selectively blocks the induction of bursting in AB, but not the excitation in the LP neuron.

![Fig. 4. Atropine blockade of AB bursting induced by GPR stimulation or puffed 5-HT. Under control conditions (A), a brief puff of 5-HT (1 mmol l\(^{-1}\)) or GPR stimulation (20 Hz) induces rhythmic bursting in the AB neuron. The induction of bursting in AB by both 5-HT and GPR disappears when atropine (0.1 mmol l\(^{-1}\)) is added to the control saline (B). The resting potential for the AB neuron is \(-63\) mV, which remains unchanged in the presence of atropine. Note that an initial hyperpolarization of the AB typically follows the puff of 5-HT. This hyperpolarization is caused in part by IPSPs (arrows) from simultaneous excitation of the LP neuron (not shown). Atropine selectively blocks the induction of bursting in AB, but not the excitation in the LP neuron.](image)

neurons usually slow down or cease their rhythmic bursting, as do all the STG neurons (Russell, 1979). As shown in Fig. 4, a brief puff of 5-HT (1 mmol l\(^{-1}\), 3 s) or a short train of GPR stimuli (20 Hz, 3 s) induced rhythmic bursting in a quiescent AB cell. 5-HT stimulation of AB/PD bursting could also be observed during recordings from the electrically coupled PD neurons (Figs 5 and 6A). The time course of the responses to GPR stimulation or 5-HT puff was somewhat different: GPR stimulation induced bursting with only a short delay, while the 5-HT puff (1 mmol l\(^{-1}\)) induced an initial hyperpolarization in AB/PD neurons before bursting began (Figs 4 and 5). In a synaptically intact preparation, part of this inhibition is due to synaptic inhibition by the

![Fig. 5. Atropine blockade of 5-HT-induced bursting in the PD neuron. (A) A brief puff of 5-HT (0.1 mmol l\(^{-1}\)) enhances rhythmic bursting in the PD neuron (control). In the intact preparation, PD responds to 5-HT with a short delay due to LP excitation and consequent synaptic inhibition. (B) This delay becomes shorter when LP is hyperpolarized (arrow) to remove the glutamatergic inhibition from LP to PD. (C) Atropine (50 \(\mu\)mol l\(^{-1}\)) reduces or blocks the PD response without affecting LP excitation. (D) The same result is seen when the LP neuron is hyperpolarized by current injection (arrow). The most hyperpolarized potentials are \(-53\) mV for PD and \(-57\) mV for LP.](image)
Multiple 5-HT receptors in the crab STG neurons

Fig. 5
LP neuron (Fig. 1). This can be seen in Figs 4 and 5, whose recordings were made without PTX in the saline, so that glutamatergic inhibition of the AB/PD neurons by LP could occur (see arrows in Fig. 4 indicating LP-evoked IPSPs; this is observed directly in
Fig. 5). When the LP neuron was hyperpolarized to remove its inhibition of the AB/PD neurons, AB/PD responded to 5-HT with a shorter delay (Fig. 5; compare the onset of PD bursting after 5-HT puff in traces A and B).

Enhancement of AB/PD bursting by GPR stimulation and 5-HT application was mimicked by bath application of two vertebrate 5-HT agonists. They are the 5-HT1 agonist 5-CT and the 5-HT2 agonist α-me-5HT (Fig. 6B,C). α-me-5HT is a selective agonist acting only on AB/PD, while 5-CT also excited the LP neuron (see below). The effects of 5-CT and α-me-5HT were dose-dependent (Tables 2 and 3). 5-CT at 5 μmol l⁻¹ and 10 μmol l⁻¹ significantly increased the bursting frequency of the AB/PD neurons by 173 % and 89 % respectively. α-me-5HT increased AB/PD bursting by 37 %, 167 % and 91 % at 1, 5 and 10 μmol l⁻¹, respectively. It appears that the effects of 5-CT and α-me-5HT were maximal at 5 μmol l⁻¹ and declined at supramaximal concentrations. No other agonists tested affected the AB/PD neurons (Table 4).

**Atropine blocks 5-HT/GPR-induced rhythmic bursting in the AB/PD neurons**

The induction and enhancement of AB/PD bursting by GPR and 5-HT was blocked by

**Table 2. Dose-dependent effects of 5-CT on PD and LP**

<table>
<thead>
<tr>
<th>[5-CT] (μmol l⁻¹)</th>
<th>Increase in PD cycling frequency (%)</th>
<th>Increase in the number of action potentials per LP burst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 4±28 (N=5; P&gt;0.05)</td>
<td>35±12 (N=4; P&lt;0.05)</td>
<td></td>
</tr>
<tr>
<td>5 173±13 (N=3; P&lt;0.05)</td>
<td>156±24 (N=4; P&lt;0.05)</td>
<td></td>
</tr>
<tr>
<td>10 89±26 (N=5; P&lt;0.05)</td>
<td>289±209 (N=5; P&lt;0.01)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.

**Table 3. Dose-dependent effect of α-me-5HT on PD**

<table>
<thead>
<tr>
<th>[α-me-5HT] (μmol l⁻¹)</th>
<th>Increase in PD cycling frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 37±58 (N=3; P&gt;0.05)</td>
<td>37±58 (N=3; P&gt;0.05)</td>
</tr>
<tr>
<td>5 167±153 (N=3; P&lt;0.05)</td>
<td>167±153 (N=3; P&lt;0.05)</td>
</tr>
<tr>
<td>10 91±110 (N=8; P&lt;0.05)</td>
<td>91±110 (N=8; P&lt;0.05)</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.
atropine (10 \mu mol l^{-1} to 0.1 mmol l^{-1}; Figs 4 and 5). For example, as shown in Fig. 4, atropine eliminated the GPR/5-HT-evoked bursting response in the AB neuron, but simultaneous excitation of the LP neuron was unaffected by atropine, as seen by the IPSPs it evoked in the AB neuron (arrows, Fig. 4). 5-HT and GPR stimulation continued to evoke bursting in the AB/PD neurons when the LP neuron was hyperpolarized to remove its synaptic inhibition of the AB/PD neurons, and this bursting was abolished by atropine (Fig. 5). Fig. 7 shows a quantitative analysis of atropine reduction of GPR- and

Table 4. Effects of serotonergic agonists on the crab stomatogastric ganglion neurons

<table>
<thead>
<tr>
<th>Agonists</th>
<th>Receptor subtypesa</th>
<th>Maximal concentration applied (\mu mol l^{-1})</th>
<th>Selected referencesb</th>
<th>LP tonic inhibition</th>
<th>AB/PD rhythmic bursting</th>
<th>Antagonized by</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCPP</td>
<td>1</td>
<td>50</td>
<td>2, 3</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5-CT</td>
<td>1, 1b, 1c</td>
<td>10</td>
<td>1, 4, 5, 6</td>
<td>–</td>
<td>+</td>
<td>Cinanserin</td>
</tr>
<tr>
<td>CGS-12066B</td>
<td>l_b</td>
<td>100</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>\alpha-me-5HT</td>
<td>2</td>
<td>10</td>
<td>3, 8, 9</td>
<td>–</td>
<td>–</td>
<td>+ Cinanserin</td>
</tr>
<tr>
<td>2-me-5HT</td>
<td>3</td>
<td>10</td>
<td>3, 8, 9, 10</td>
<td>+</td>
<td>–</td>
<td>– Gramine</td>
</tr>
<tr>
<td>1-Phenylbiguanide</td>
<td>3</td>
<td>100</td>
<td>11</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

a5-HT receptor subtypes in vertebrates.

b(1) Peroutka (1988); (2) Lucki et al. (1989); (3) Holophean et al. (1990); (4) Schmidt and Peroutka (1989); (5) Roberts et al. (1988); (6) Rasmussen and Aghajanian (1990); (7) Neale et al. (1987); (8) Mawe et al. (1986); (9) Richardson et al. (1985); (10) Yankel and Jackson (1988); (11) Ireland and Tyers (1987).

c+ indicates that the agonist mimics the effects of GPR and 5-HT; – indicates that the agonist has no effect.

Fig. 7. A plot of pyloric cycle frequency enhancement by 5-HT pressure ejection and its blockade by atropine. Values were collected from four experiments. A paired Student’s t-test shows that 5-HT significantly (P<0.05) enhances the PD bursting frequency (marked with an asterisk). The response to 5-HT is blocked by atropine (0.1 mmol l^{-1}).
5-HT-induced AB/PD bursting. A paired Student’s t-test indicates a significant blockade by atropine. Thus, atropine selectively blocks GPR- and 5-HT-induced rhythmic bursting excitation of the AB neuron. Atropine did not block either the responses of other neurons to 5-HT (Table 5, Fig. 5) or the responses of AB/PD to the 5-HT agonists 5-CT and α-me-5HT (not shown).

The muscarinic agonist pilocarpine and the monoamine dopamine also induce
rhythmic bursting in the AB/PD neurons in the lobster *Panulirus interruptus* and in the crab *C. borealis* (Marder, 1987; Harris-Warrick and Flamm, 1987). In contrast to its complete blockade of GPR- and 5-HT-induced AB/PD bursting, atropine (0.1 mmol l\(^{-1}\)) only slightly reduced pilocarpine-induced bursting (Fig. 8) and did not affect dopamine-induced AB/PD bursting (data not shown). Thus, atropine at these concentrations is a relatively selective 5-HT receptor antagonist and does not effectively block muscarinic cholinergic receptors or cause a non-selective inhibition of the bursting mechanism.

Cinanserin blocks AB/PD bursting evoked by GPR, 5-HT, 5-CT and \(\alpha\)-me-5HT

The vertebrate 5-HT\(_{1c,2}\) antagonist cinanserin (10–20 \(\mu\)mol l\(^{-1}\)) blocked the enhancement of AB/PD bursting by GPR stimulation and 5-HT (Fig. 6A) and by bath application of the 5-HT agonists 5-CT and \(\alpha\)-me-5HT (Fig. 6B,C). This blockade was completely reversed upon washout. Cinanserin by itself had no effect on the AB/PD baseline activity. Other vertebrate 5-HT antagonists (except atropine) had no effects on 5-HT- or GPR-induced rhythmic bursting of the AB/PD neurons (Table 5).

To show that cinanserin is a selective antagonist for 5-HT, we tested its effects on AB/PD bursting induced by the muscarinic agonist pilocarpine. Fig. 9 shows that cinanserin had no effect on pilocarpine-induced AB/PD bursting.

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Table 5. Effects of serotonergic antagonists on the crab STG neurons

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>Receptor subtypes(^a)</th>
<th>Maximal concentration applied ((\mu)mol l(^{-1}))</th>
<th>Selected references(^b)</th>
<th>PY tonic inhibition</th>
<th>LP excitatory</th>
<th>PD/AB rhythmic bursting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metergoline</td>
<td>1(_{c,2}), 2</td>
<td>100</td>
<td>1, 2, 3, 4</td>
<td>-(\sim)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sipiperone</td>
<td>1(_{c,2})</td>
<td>10</td>
<td>3, 4, 5, 6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methysergide</td>
<td>1(_c), 2</td>
<td>10</td>
<td>7, 8, 9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cinanserin</td>
<td>2, 1(_c)</td>
<td>20</td>
<td>1, 7</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mianserin</td>
<td>2, 1(_c)</td>
<td>10</td>
<td>3, 4, 5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pirenperone</td>
<td>2</td>
<td>20</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cyproheptadine</td>
<td>2</td>
<td>100</td>
<td>1, 11</td>
<td>-</td>
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<td>0</td>
</tr>
<tr>
<td>MDL72222</td>
<td>3</td>
<td>100</td>
<td>3, 4, 5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zacopride</td>
<td>3</td>
<td>20</td>
<td>3, 4</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Metoclopramide</td>
<td>3</td>
<td>100</td>
<td>11, 12</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Atropine</td>
<td>u</td>
<td>100</td>
<td>13, 14</td>
<td>-</td>
<td>-</td>
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\(^a\)5-HT receptor subtypes in vertebrates. Atropine and gramine are classified as undetermined subtypes.

\(^b\)(1) McCall and Aghajanian (1980); (2) White and Neuman (1983); (3) Peroutka (1988); (4) Schmidt and Peroukta (1989); (5) Hololehan *et al.* (1990); (6) Felder *et al.* (1990); (7) Proudfit and Anderson (1973); (8) Roberts *et al.* (1988); (9) McCall and Aghajanian (1979); (10) Barbeau and Rossignol (1990); (11) Yakel *et al.* (1988); (12) Yakel and Jackson (1988); (13) Gaddum and Picarelli (1957); (14) Gerschenfeld and Stefani (1966); (15) Colpaert *et al.* (1982).

\(^c\)- and + indicate non-blockade and blockade, respectively; 0 means the drug was not tested.
5-HT and 5-CT mimic the effects of GPR on the LP neuron

GPR stimulation and 5-HT application evoked tonic depolarization and high-frequency spiking in the LP neuron (Figs 5, 10A). During the 5-HT puff or GPR stimulation, LP fired a tonic burst of action potentials at high frequency that effectively inhibited oscillations in the pacemaker AB/PD neurons (see Figs 4 and 5; Katz and Harris-Warrick, 1990a). Following this, the LP fired at high frequency but was rhythmically inhibited by the AB/PD group.

Fig. 9. The effects of cinanserin on pilocarpine-induced AB/PD bursting. The muscarinic agonist pilocarpine (0.1 mmol l\(^{-1}\)) induces rhythmic bursting in AB/PD, as recorded in the PD neuron (B). Cinanserin (10 μmol l\(^{-1}\)) does not affect this response (C). The most hyperpolarized potentials of PD are −60 mV (A), −57 mV (B and C) and −54 mV (D).
LP became tonically active in the absence of AB/PD bursting. Under these conditions, the 5-HT₁ agonist 5-CT depolarized LP and increased its tonic spiking in a dose-dependent manner with a maximal effect at 10 μmol L⁻¹ (Table 2; Fig. 10B). This is different from the actions of 5-CT on AB/PD bursting, which were maximal at 5 μmol L⁻¹ and declined at higher concentrations (Table 2). In a weakly oscillating preparation, 5-CT (10 μmol L⁻¹) enhances the plateau ability of LP and increases the number of spikes per burst. This effect is blocked by cinanserin (10 μmol L⁻¹). The most hyperpolarized potentials of LP are −56 mV (control and 5-CT), −59 mV (5-CT+cinanserin) and −57 mV (wash).

Fig. 10. 5-HT and 5-CT excitation of LP and the blockade by cinanserin. (A) GPR stimulation (20 Hz) and a brief puff of 5-HT (0.1 mmol L⁻¹) induce tonic excitation (depolarization and increased action potentials). Cinanserin (20 μmol L⁻¹) blocks these responses. The resting potential of LP is −250 mV. (B) 5-CT mimics the effect of 5-HT. In the absence of AB/PD bursting, 5-CT (10 μmol L⁻¹) depolarizes (from −40 mV to −35 mV) and enhances tonic spiking in LP. (C) In a slowly oscillating preparation, 5-CT (10 μmol L⁻¹) enhances the plateau ability of LP and increases the number of spikes per burst. This effect is blocked by cinanserin (10 μmol L⁻¹). The most hyperpolarized potentials of LP are −56 mV (control and 5-CT), −59 mV (5-CT+cinanserin) and −57 mV (wash).
Cinanserin also blocks LP tonic excitation evoked by GPR, 5-HT and 5-CT

Cinanserin (1–20 μmol l^{-1}) abolished both GPR-evoked and 5-HT-induced LP activity (Fig. 10A). Cinanserin also blocked tonic LP excitation evoked by the 5-HT agonist 5-CT (Fig. 10 B,C). None of the other antagonists, including atropine (Figs 4 and 5), blocked the response of the LP neuron to GPR, 5-HT or 5-CT (Table 5).

Discussion

GPR cells use 5-HT as a slow neuromodulator

Motor patterns generated by the crustacean STG can be altered by a variety of neuromodulators that are released by identified neurons (for reviews, see Katz and Harris-Warrick, 1990b; Harris-Warrick et al. 1992). In the crab, the GPR cells are mechanosensory neurons that appear to use both ACh and 5-HT as co-transmitters. GPR stimulation evokes rapid EPSPs with a typical nicotinic cholinergic pharmacological profile as well as a variety of slow modulatory responses (Katz and Harris-Warrick, 1989, 1990a). We have studied three distinct slow modulatory responses to GPR stimulation observed in different pyloric neurons: (1) tonic inhibition in the PY neurons; (2) induction and enhancement of rhythmic bursting in the AB/PD neurons; and (3) prolonged tonic excitation in the LP neuron (Katz and Harris-Warrick, 1989, 1990a). A number of other STG neurons also show slow modulatory responses to GPR stimulation; these responses include induction of plateau potentials (Katz and Harris-Warrick, 1989; Kiehn and Harris-Warrick, 1992) as well as responses that appear to be similar to one of these three classes of response (Katz and Harris-Warrick, 1989), but they were not examined in this study. It has been proposed (Katz and Harris-Warrick, 1989) that these slow modulatory responses are induced by the release of 5-HT from the GPR cells. In support of this, the slow responses are not blocked by a combination of muscarinic (except atropine; see below) and nicotinic cholinergic antagonists (Katz and Harris-Warrick, 1989, 1990a; Katz, 1989). Further, the slow effects of GPR stimulation are mimicked by either bath application (Katz and Harris-Warrick, 1989, 1990a) or pressure ejection (this paper; Kiehn and Harris-Warrick, 1992) of 5-HT. In this paper, we show that a set of 5-HT agonists selectively mimics the different effects of GPR and 5-HT on different neurons. Moreover, the specific responses to GPR stimulation and application of 5-HT or its agonists have the same profile of sensitivity to a set of pharmacological 5-HT antagonists. This provides further support for our hypothesis that the GPR cells use 5-HT to induce slow modulatory responses in STG neurons.

Evidence for serotonergic modulation via multiple 5-HT receptors

Our experiments used pharmacological agonists and antagonists of 5-HT to determine whether there is more than one type of 5-HT receptor evoking the different responses of identified pyloric neurons to 5-HT and GPR stimulation. Our results show that 5-HT exerts its modulatory effects via multiple receptors that are pharmacologically distinct on the different STG neurons.

GPR stimulation or 5-HT application elicits a transient inhibition of the PY neurons. Katz and Harris-Warrick (1989, 1990a) showed that this inhibition is a direct effect of the
GPR cell onto PY, rather than an indirect effect mediated by excitation of other STG neurons. The inhibition of PY was selectively mimicked by 2-me-5HT, but not by any other agonists that we tested (Fig. 3; Table 4). Gramine can block or reduce the PY inhibition induced by GPR stimulation as well as by application of 5-HT or 2-me-5HT (Figs 2 and 3). Gramine itself had no effect on the baseline activity of the PY neuron, and it had no effect on the responses of the other neurons to 5-HT and its agonists or to GPR stimulation. None of the other drugs we tested antagonized the inhibitory response of PY to 5-HT (Table 5). Thus, we conclude that 2-me-5HT is a selective agonist and gramine a selective antagonist for the 5-HT receptors that cause PY inhibition in the STG.

Serotonin and GPR stimulation evoke or enhance rhythmic bursting in the electrically coupled AB/PD neurons (Figs 4, 5 and 6A). Two 5-HT agonists, 5-CT and α-me-5HT, mimic the effects of 5-HT and GPR on the AB/PD neurons (Fig. 6B,C). Cinanserin and atropine antagonized burst enhancement of the AB/PD neurons by 5-HT and GPR (Figs 5, 6A and 7). Cinanserin (but not atropine) also blocked the effects of 5-CT and α-me-5HT on AB/PD bursting (Fig. 6B,C). Moreover, cinanserin did not affect AB/PD bursting induced by dopamine or the muscarinic agonist pilocarpine (Fig. 9), suggesting that it is selective for 5-HT receptors.

Atropine is typically considered as a muscarinic cholinergic antagonist. However, in the crustacean STG, atropine is a weak antagonist at muscarinic receptors, causing complete block only at 1 mmol l⁻¹ (Marder and Eisen, 1984; Katz, 1989). In contrast, atropine blocked GPR- and 5-HT-induced AB/PD bursting at 10–100 μmol l⁻¹ (Figs 4 and 5; Katz and Harris-Warrick, 1989), at which concentration it only has a weak effect on muscarinic receptors activated by the muscarinic agonist pilocarpine (Fig. 8). In addition, atropine did not antagonize any of the other 5-HT- or GPR-induced responses in the pyloric neurons (Table 5). Thus, in the STG, atropine at low concentrations appears to act as a relatively selective antagonist of a subset of the 5-HT receptors inducing bursting in the AB/PD neurons. Atropine also blocks 5-HT receptors in guinea pig ileum (Gaddum and Picarelli, 1957), in molluscan neurons (Gerschenfeld and Stefani, 1966) and in leech neurons (Smith and Walker, 1975), so there are precedents for this effect. However, atropine at the concentrations we used failed to block AB/PD bursting induced by the 5-HT agonists 5-CT or α-me-5HT. We do not understand this result. It is unlikely that atropine is acting in some non-selective manner, since it abolished GPR- and 5-HT-evoked AB/PD bursting but had little or no effect on pilocarpine- and dopamine-induced bursting. It is possible that the AB/PD neurons possess multiple 5-HT receptors with differential selectivity for 5-CT, α-me-5HT, cinanserin and atropine, but their interactions would have to be complex to explain this result.

Katz and Harris-Warrick (1989, 1990a) demonstrated that GPR stimulation directly excites the LP neuron. We have shown that a brief puff of 5-HT and bath-applied 5-CT mimicked the effect of GPR stimulation on LP activity (Fig. 10). Cinanserin (20 μmol l⁻¹) blocked the tonic excitation induced by GPR stimulation, by puffed 5-HT or by bath-applied 5-CT. However, atropine, which blocked the AB/PD response, did not block the LP response (Fig. 5; Table 5). Further, α-me-5HT, which mimics the action of 5-HT on the AB/PD neurons, had no effect on the LP neuron (Table 4). Thus, the 5-HT receptor(s) mediating tonic excitation in the LP neuron appear not to be identical to those
mediating rhythmic burst induction in the AB/PD neurons. Either these neurons express completely different 5-HT receptors that are all capable of being blocked by cinanserin or the LP neuron shares a cinanserin-sensitive receptor with the AB/PD neurons, but differs in its other 5-HT receptors.

**Comparative views of vertebrate and invertebrate 5-HT receptors**

The characterization of 5-HT receptor types in vertebrate nervous systems is quite advanced as a result of the large number of selective agonists and antagonists that exist (Peroutka, 1991). However, little is known about 5-HT receptor types in invertebrates. Using available 5-HT agonists and antagonists developed from vertebrate studies, we hoped to begin to identify 5-HT receptor subtypes in a well-defined invertebrate neural network in which cells can be readily identified and the functions of multiple receptor types can be studied.

Several recent studies have shown that certain vertebrate 5-HT antagonists block 5-HT receptors in crustaceans and insects. 5-HT stimulates the Na+/K+-ATPase that is involved in the retraction of the proximal pigment in crayfish photoreceptor cells. High concentrations of methysergide and cyproheptadine (1 mmol l\(^{-1}\)) block this effect of 5-HT, presumably through antagonism of specific 5-HT receptors (Aréchiga et al. 1990; Frixione and Hernández, 1989). In *Rhodnius prolixus*, mianserin, methysergide and cyproheptadine block a 5-HT-induced increase in intracellular cyclic AMP concentration in adult anterior midgut and Malpighian tubules (Barrett and Orchard, 1990). The 5-HT\(_{2}\) antagonists ketanserin and spiperone also block 5-HT-induced fluid secretion in the Malpighian tubules (Maddrell et al. 1991). In the mollusc *Aplysia californica*, 5-HT increases the efficacy of synaptic transmission between siphon sensory cells and motor neurons. The 5-HT antagonist cyproheptadine blocks spike-broadening and the enhancement of synaptic facilitation induced by 5-HT or sensitizing stimulation of the tail (Mercer et al. 1991). In central neurons of the snail *Helix pomatia*, several 5-HT agonists (including 5-CT and \(\alpha\)-me-5HT) and antagonists (including cinanserin and MDL 72222) were found to act on specific 5-HT receptors (Vehovszky and Walker, 1991; Vehovszky et al. 1992). We have demonstrated that the vertebrate 5-HT\(_{1c/2}\) antagonist cinanserin blocks 5-HT receptors and that three 5-HT agonists selectively mimic 5-HT-induced responses in crab STG neurons.

Despite the fact that some vertebrate agonists and antagonists work well on invertebrate receptors, there is increasing evidence that 5-HT receptors in invertebrate nervous systems are strikingly different from the well-characterized 5-HT receptor subtypes of vertebrates. These differences are both pharmacological and functional (Smith and Walker, 1975; Vehovszky and Walker, 1991). Tables 4 and 5 show that classical vertebrate receptor subtype agonists and antagonists cannot define the crab 5-HT receptors. For example, the 5-HT\(_{3}\) agonist 2-me-5HT inhibits the PY neurons, but another 5-HT\(_{3}\) agonist, 1-phenylbiguanide, does not. Moreover, two 5-HT\(_{3}\) antagonists (MDL 72222 and zacopride) fail to block the effects of 5-HT or 2-me-5HT on the PY neurons, while the non-classical antagonist gramine is very effective. The 5-HT\(_{1a,1b}\) agonist 5-CT excites the AB/PD and LP neurons, and this effect is antagonized by the 5-HT\(_{1c/2}\) antagonist cinanserin, but not by other 5-HT\(_{1c/2}\) antagonists (methysergide and
mianserin). In fact, the great majority of vertebrate 5-HT antagonists we tested had no detectable effect on the crab 5-HT receptors (Table 5).

In addition, the vertebrate receptor subtypes evoke typical membrane electrical responses in their neurons. In vertebrates, 5-HT$_1$ agonists typically evoke hyperpolarization via a G-protein-coupled second-messenger mechanism (Andrade and Chaput, 1991b). In contrast, the 5-HT$_1$ agonist 5-CT excites the crab AB/PD and LP neurons. The vertebrate 5-HT$_3$ receptor is a ligand-gated ion channel mediating rapid depolarization (Yankel and Jackson, 1988; Derkach et al. 1989), while the 5-HT$_3$ agonist 2-me-5HT slowly inhibits the crab PY neurons.

These results show that, although certain vertebrate 5-HT agonists and antagonists can be used to identify multiple 5-HT receptor subtypes in the crab STG neurons, these receptors are sufficiently different that the vertebrate subtype classifications cannot be used to name the crab receptors and other invertebrate receptors (Walker, 1985). Further research will be needed before a rational invertebrate 5-HT receptor classification can be made.

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Multiple 5-HT receptors in the crab STG neurons


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