Real-Time Measurements of Synaptic Autoinhibition Produced by Serotonin Release in Cultured Leech Neurons

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Cercós MG, De-Miguel FF, Trueta C. Real-time measurements of synaptic autoinhibition produced by serotonin release in cultured leech neurons. J Neurophysiol 102: 1075–1085, 2009. First published June 7, 2009; doi:10.1152/jn.00107.2009. We studied autoinhibition produced immediately after synaptic serotonin (5-HT) release in identified leech Retzius neurons, cultured singly or forming synapses onto pressure-sensitive neurons. Cultured Retzius neurons are isopotential, thus allowing accurate recordings of synaptic events using intracellular microelectrodes. The effects of autoinhibition on distant neuropolar presynaptic endings were predicted from model simulations. Following action potentials (APs), cultured neurons produced a slow hyperpolarization with a rise time of 85.4 ± 5.2 ms and a half-decay time of 252 ± 17.4 ms. These inhibitory postpotentials were reversibly abolished by the iontophoretic application of 5-HT and became depolarizing after inverting the transmembranal chloride gradient by using microelectrodes filled with potassium chloride. The inhibitory postpotentials were reversibly abolished in the absence of extracellular calcium and absent in reserpine-treated neurons, suggesting an autoinhibition due to 5-HT acting on autoreceptors coupled to chloride channels. The autoinhibitory responses increased the membrane conductance and decreased subsequent excitability. Increasing 5-HT release by stimulating with trains of ten pulses at 10 or 30 Hz produced 23 ± 6 and 47 ± 2% of AP failures, respectively. These failures were reversibly abolished by the serotoninergic antagonist methysergide (140 μM). Moreover, reserpine-treated neurons had only 5 ± 4% of failures during trains at 10 Hz. This percentage was increased to 35 ± 4% by iontophoretic application of 5-HT. Increases in AP failures correlated with smaller postsynaptic currents. Model simulations predicted that the autoinhibitory chloride conductance reduces the amplitude of APs arriving at neuropilar presynaptic endings. Altogether, our results suggest that 5-HT autoinhibits its subsequent release by decreasing the excitability of presynaptic endings within the same neuron.

INTRODUCTION

Serotonin (5-HT), a major neuromodulator of behavior in vertebrates and invertebrates, inhibits the electrical activity of serotoninergic neurons in a phenomenon known as autoinhibition (Aghajanian et al. 1987; Heinrich et al. 1999). Experimental evidence combining electrical stimulation of serotoninergic neurons with pharmacological manipulations has shown that increasing the levels of extracellular 5-HT or applying 5-HT agonists decreases the firing frequency of serotoninergic neurons, whereas the application of antagonists for 5-HT autoreceptors increases their firing rate (Chaput et al. 1986; Fornal et al. 1994; Gobert et al. 1995; O’Connor and Kruk 1991; Rogawski and Aghajanian 1981; Trulson and Crisp 1986; Trulson and Frederickson 1987). Consistently, 5-HT release evoked by extracellular stimulation is reduced by serotoninergic autoreceptor agonists and increased by serotoninergic autoreceptor antagonists (Baumann and Waldmeier 1984; Birthelmer et al. 2007; Bonanno et al. 1986; Chaput et al. 1986; Davidson and Stamford 1995; Galzin et al. 1992; Göthert 1990; Piñeyro et al. 1995; Rollema et al. 1997; Rutz et al. 2007; Starkey and Skingle 1994; Stauderman and Jones 1986; Wichmann et al. 1989). How the ongoing 5-HT release affects the presynaptic electrical activity and the subsequent release of 5-HT have been difficult to study directly due to the distance between the recording sites and the presynaptic endings. These questions have been addressed here by recording autoinhibitory responses from identified serotonergic Retzius neurons in culture, after their isolation from the CNS of the leech Hirudo medicinalis.

Retzius neurons are the major serotoninergic neurons in the leech CNS, containing around 50% of the total 5-HT in the animal (Coggshall 1972). Upon visual identification they can be individually isolated and plated in culture, where they maintain their identity (Fuchs et al. 1981) and continue synthesizing, storing, and releasing 5-HT (Henderson et al. 1983), either from unidirectional presynaptic terminals onto pressure sensory neurons (Ready and Nicholls 1979; for review see Fernandez de Miguel and Drapeau 1995) or from the soma (De-Miguel and Trueta 2005; Trueta et al. 2003). Synaptic and somatic release of 5-HT have different dependencies on the firing frequency (De-Miguel and Trueta 2005). Whereas synaptic release is activated by single action potentials (APs) (Fuchs et al. 1982), somatic secretion occurs at high firing frequencies (Trueta et al. 2003). Since presynaptic release sites are formed very near the soma, the somatic recordings contain faithful representations of the electrical events occurring at the synapses (Fuchs et al. 1982). For these reasons, Retzius neurons in culture provide an excellent preparation to study serotoninergic autoinhibition occurring at presynaptic terminals with good space and time resolution.

Autoinhibition produced by 5-HT release was studied from hyperpolarizing postpotentials produced in cultured Retzius neurons after their stimulation with intracellular current pulses. Further evidence as to how autoinhibition affects the neuronal excitability and the release of 5-HT was obtained from simultaneous pre- and postsynaptic recordings from synapses formed in culture between Retzius and pressure-sensitive neurons. Modeling was used to predict how 5-HT release might act at presynaptic endings of branches within the neuropile.
METHODS

Isolation and culture of neurons

Experiments were performed using identified Retzius neurons isolated from the CNS of adult leeches Hirudo medicinalis. Animals were handled and maintained according to the National Institutes of Health Guide for Care and Use of Laboratory Animals, with approval of the local animal care committee. The isolation procedure has been described elsewhere (Dietzel et al. 1986). In brief, leeches were anesthetized by immersion in 8% ethanol, nerve cords were dissected, and ganglion capsules were opened to expose the cell somata. Ganglia were kept in Leibovitz L-15 culture medium (Gibco, Gaithersburg, MD), supplemented with 6 mg/ml glucose, 0.1 mg/ml gentamicin, and 2% heat-inactivated fetal bovine serum (Gibco); they were incubated for 1 h with 2 mg/ml collagenase-dispase (Roche, Mannheim, Germany) on a rocking plate. Retzius and pressure sensory (P) neurons were individually isolated by suction through a glass pipette, rinsed in sterile L-15, and plated on culture dishes (Falcon, Franklin Lakes, NJ) coated with concanavalin-A (Sigma, St. Louis, MO). For synaptic transmission measurements, Retzius neurons were plated placing their stump in contact with the soma of a P neuron (Dietzel et al. 1986). Experiments were done at 18–20°C, 1–7 days after plating.

In some experiments, reserpine (Sigma; 1 μM diluted from a 500 μM stock solution dissolved in methanol) was added to the culture medium at the time of plating and was left for 7 days before electrophysiological recordings were carried out (Drapeau et al. 1989; O’Gara et al. 1991). The final concentration of methanol in the culture medium was 0.2%. As a negative control for this experiment, some neurons were incubated with only 0.2% methanol in the culture medium.

Intracellular recording and stimulation

Microelectrodes for intracellular recordings and stimulation were made with borosilicate glass capillaries with a 1-mm OD and a 0.75-mm ID, pulled in a P97 puller (Sutter Instruments, Novato, CA). Electrodes with resistances of 18–25 MΩ, filled with 4 M potassium acetate (KAc) were used. For some experiments, electrodes were filled with 3 M potassium chloride (KCl) to invert the transmembranal chloride gradient. An Axoclamp-2B amplifier (Axon Instruments, Union City, CA) in bridge mode was used. Stimulation consisted of trains of APs produced by intracellular injection of 10-ms current pulses at different frequencies using an S88 stimulator connected to an SIU5 isolation unit (Grass Instruments, West Warwick, RI). Some recordings were made in single-electrode discontinuous voltage-clamp conditions. Although APs during the stimulation train could not be clamped in these conditions, the direction of the current at the end of a stimulation train could be observed (see arrows in Fig. 1, C1 and C2). Electrical recordings were acquired by an A/D board Digidata 1320 (Axon Instruments) at a sampling frequency of 20 kHz using pCLAMP9 software (Axon Instruments) and stored in a PC.

To block the AP-evoked release of 5-HT, in some experiments the neurons were perfused with leech saline solution in the absence of calcium (Katz and Miledi 1967), which was replaced by 2 mM magnesium (Mg2+). This solution was preferred over that with an increase in the Mg2+ concentration because in our experience, high concentrations of magnesium visibly damage cultured leech neurons, which under electron microscopy appear morphologically disrupted, with swelled and excentric dense-core vesicles, and the AP rapidly deteriorates (Trueta et al. 2003; see also Henderson et al. 1983).

To measure the effect of externally applied 5-HT on the permeability of the membrane, hyperpolarizing intracellular current pulses of amplitude between 120 and 300 pA and 1-s duration were applied to the neurons at a frequency of 0.5 Hz and the voltage response was recorded in discontinuous current-clamp conditions. The amplitude of the hyperpolarizing current pulses was kept at these small values to avoid nonlinear voltage responses. After 10 s of control recording, an iontophoretic 5-HT pulse was delivered before each hyperpolarizing pulse for 5 s. Hyperpolarizing pulses then continued without 5-HT application to observe recovery. Changes in the input resistance were estimated from the amplitude of the steady-state voltage response to hyperpolarizing current pulses. The values shown in Fig. 4 are the percentage of the voltage-response amplitude measured before 5-HT application.

Serotonin iontophoresis

We used microelectrodes similar to those used for intracellular recordings, filled with 150 mM 5-hydroxytryptamine (5-HT) HCl application.

![Fig. 1. A slow chloride-dependent response follows electrical activity in cultured Retzius neurons. A and B: recordings of the membrane potential of Retzius neurons stimulated with a single depolarizing current pulse of 10 ms (A) or trains of 10 pulses at 30 Hz (B) using an intracellular electrode filled with potassium acetate (KAc, top traces) or with potassium chloride (KCl, bottom traces). Single action potentials (APs) and trains of impulses were followed by autoinhibitory postpotential, which under electron microscopy appear morphologically disrupted, with swelled and excentric dense-core vesicles, and the AP rapidly deteriorates (Trueta et al. 2003; see also Henderson et al. 1983).](http://jn.physiology.org/ftp/jn10021/jn10021-0012fig1.jpg)
(Sigma) dissolved in leech physiological solution (in mM: NaCl 120; KCl 4; CaCl₂ 1.8; Tris-maleate 10; pH 7.4). To retain 5-HT in the electrode, a negative 1-nA DC current was applied by using an intracellular amplifier (Getting USA). For 5-HT iontophoresis, positive current pulses of 10-nA amplitude and 20-ms duration were applied to the electrode, whose tip was placed near the soma of the Retzius neuron. Electrodes filled only with physiological solution or negative current pulses failed to elicit the responses seen by application of 5-HT (not shown).

Excitability studies

To study how 5-HT released from Retzius neurons affects their excitability, we analyzed the number of APs in response to trains of intracellular current pulses. The amplitude of the intracellular current pulses was the minimum necessary to produce a single AP with each current pulse when delivered at a frequency of 1 Hz. The same current amplitude was then used in trains at different frequencies in the same neuron. Upon increases in the stimulation frequency, neurons failed to produce an AP in response to every current pulse. The subthreshold depolarizations were defined as AP failures. The percentage of failures was calculated by taking the total number of current pulses in the train as 100%. In a series of experiments, we used the nonelective 5-HT antagonist methysergide (Sigma; 140 μM), diluted from a 4.2 mM stock solution dissolved in physiological solution. Ten minutes after its application, it was washed out by perfusion with leech physiological solution to see recovery.

Synaptic transmission measurements

To study how serotonergic autoinhibition affects further release, we used synapses formed between Retzius and P neurons in culture (Dietzel et al. 1986). Presynaptic Retzius neurons were stimulated with a test pulse of depolarizing current followed after 5 s by a train of ten pulses, using KAc-filled electrodes. Synaptic currents were recorded from the P cell under single-electrode voltage-clamp conditions using a holding potential of −50 mV. CsCl-filled electrodes were used in the P cells to reverse and amplify the inhibitory synaptic potentials, since the resting potential is close to the chloride equilibrium potential (Fuchs et al. 1982), and to increase the input resistance. Capacitative coupling between the stimulating and recording electrodes was reduced by interposing a grounded shield. During the recording, neurons were kept in Leibowitz-15 medium and synaptic currents were increased by raising the calcium concentration from 1.8 to 10 mM (Dietzel et al. 1986).

Since AP failures in Retzius neurons reduce the amount of 5-HT release during the train, to compare the release in these conditions with release in the absence of autoinhibition, a second trial was made in the same neuronal pairs using presynaptic current pulses of increased amplitude to ensure the firing of an AP in response to each current pulse along the trains. This strategy was used to overcome autoinhibition and compare the release with and without autoinhibition, while avoiding the use of pharmacological tools that would also block the postsynaptic response, since there are not specific antagonists of presynaptic autoreceptors in these neurons.

Statistical analysis

Results are presented as means ± SE. Data were tested for normality and for variance homogeneity using Kolmogorov–Smirnov “z” test and Levene’s test, respectively. Student’s t-test was used to compare the mean of only two independent data groups. One-way ANOVA was used for comparison of more than two groups, applying a repeated-sample test beforehand in the case of dependent data groups. For comparison of the percentage of failures in the absence and presence of methysergide, where data included several zeros, the nonparametric Friedman test was used. The synaptic release index in the presence and absence of failures in the same neuronal pair was compared by using the nonparametric Wilcoxon’s t-test for dependent samples. In all cases, differences were considered significant when P < 0.05.

Modeling

Realistic mathematical models of Retzius neurons, either in culture or in the ganglion, were based on electrophysiological and morphological experimental evidence (Garcia-Perez et al. 2004; M Vargas and FF De-Miguel, unpublished observations). The model was designed by using the simulator NEURON (Hines and Canevale 2003). The soma was 100 μm in diameter and for simulations in culture it was connected to a cylindrical stump that was 60 μm in length and 20 μm in diameter. For simulations of responses in neuropil synaptic terminals, the soma was connected to a primary axon that was 200 μm in length and 20 μm in diameter, to which neurites were added. Based on morphological analysis of Retzius neurons filled with horseradish peroxidase or with Lucifer yellow (Vargas and De-Miguel, unpublished observations), neurites were either 100 μm long without branches (Garcia-Perez et al. 2004) or 75 μm long with three 75-μm branches connected at the tip of the primary branch. The diameters of the neurites and their branches were 1 μm. Presynaptic terminals were added at the tips of the stump or the neurites to simulate the 5-HT autoinhibitory presynaptic responses.

The membrane active currents producing the APs were as described by Stewart et al. (1989a) and were modeled as in Baccus (1998) and Vargas and De-Miguel (2009). The voltage-dependent conductances were a Na⁺ conductance, a delayed rectifier K⁺ conductance (Kd del), a Ca²⁺-dependent K⁺ conductance, a high-voltage-activated Ca²⁺ conductance, and a nonspecific leak conductance. The conductances were calculated by using Hodgkin–Huxley-type equations with parameters determined from voltage-clamp data (Johansen and Kleinhaus 1990). The chloride conductance induced by 5-HT release was simulated by inserting an inhibitory synapse with a rise time of 80 ms, a decay time constant of 100 ms, and ECl = −66 mV (Munsch and Schlue 1993). The activation of the synapse was synchronized with the production of each AP and variable degrees of facilitation were added on subsequent impulses, based on the rate of facilitation of postsynaptic currents recorded under voltage-clamp conditions of cultured neurons stimulated at 10 Hz (Stewart et al. 1989b; Trueta and De-Miguel, unpublished observations). The integration time step (dt) was 25 μs. To test the accuracy of our model, the predicted shapes of the APs, the chloride-dependent responses, and the sequence of APs produced by a 10-Hz train in cultured synapses were compared with the model predictions (see Fig. 7).

RESULTS

Autoinhibitory responses of serotonergic Retzius neurons

Following the peak of the hyperpolarizing postpotential characteristic of the AP of Retzius neurons in the ganglion and in culture (Fuchs et al. 1981; Nichols and Baylor 1968; Stewart et al. 1989a; Velazquez-Ulloa et al. 2003; Yang and Kleinhaus 1984; see gray arrow in Fig. 1A1, inset), our recordings consistently displayed a slow hyperpolarization (Fig. 1A1), with amplitudes ranging from −1 to −11.6 mV at a resting potential of −60 mV (average amplitude of 6.5 ± 1.2 mV), a time to peak ranging from 51 to 130 ms (average of 85.4 ± 5.2 ms), and a long half-decay time ranging from 116 to 430 ms (average of 252 ± 17.4 ms; n = 28). This inhibitory response was present in single and in paired Retzius neurons in culture, but has been difficult to detect from recordings of neurons kept in the ganglion, thus suggesting that it may occur in presynaptic terminals distant from the recording sites. In
addition, its similarities to the responses of Retzius neurons to iontophoretic application of 5-HT (Drapeau et al. 1989; Liu and Nicholls 1989; Munsch and Schlue 1993; see insets in Fig. 1, B1 and B2) suggest that they are autoinhibitory responses to 5-HT acting on autoreceptors, which in situ are present in distant presynaptic terminals, although in our culture conditions occur electrically close to the recording electrode. These slow hyperpolarizing responses became the main focus of this study and we will refer to them as autoinhibitory postpotentials.

The amplitude and duration of the autoinhibitory postpotential was increased during subsequent stimulation, becoming more prominent after trains of 10 impulses at frequencies between 10 and 30 Hz (Fig. 1B1). The average amplitude of the autoinhibitory postpotential following trains at 30 Hz (−6.9 ± 1.6 mV; n = 6) was not significantly different from that following a single AP. However, its duration was significantly longer after trains of 10 impulses, reaching a time to peak of 108 ± 21 ms and a half-decay time of 407 ± 128 ms. There were no significant differences when the trains had frequencies of 10 or 30 Hz. The changes in the autoinhibitory postpotential in response to trains occurred without any effects on the amplitude and duration of the APs and correlated well with the expected increases in the release of 5-HT (De-Miguel and Trueta 2005; Stewart et al. 1989b), thus suggesting that these slow hyperpolarizations were produced by 5-HT being released by the Retzius neuron.

To study this possibility, we first explored whether the autoinhibitory postpotential was produced by chloride currents, like those produced by 5-HT in these neurons (Drapeau et al. 1989; Liu and Nicholls 1989; Munsch and Schlue 1993; see insets in Fig. 1, B1 and B2). In recordings made with intracellular electrodes filled with 3 M KCl to reverse the transmembranal chloride gradient, the autoinhibitory postpotential turned into a depolarization (Fig. 1, A2 and B2; n = 8), which reached an amplitude of 15.41 ± 2.71 mV after trains of ten impulses at 30 Hz (Fig. 1B2), although it kept the same time course already described. Under these conditions, 5-HT applied by iontophoresis also produced a depolarization (Fig. 1B2, inset). Consistently, when the chloride gradient was physiological, a train of impulses evoked an outward transmembranal chloride current that could be recorded under single-electrode voltage-clamp conditions (arrow in Fig. 1C1). The direction of this current was reversed by the use of KCl-filled electrodes (arrow in Fig. 1C2), thus supporting the hypothesis that a chloride current underlies the autoinhibitory postpotentials.

Because in physiological conditions E_{Cl} is close to the resting membrane potential, in some neurons it was difficult to observe the autoinhibitory postpotential using electrodes filled with KAc. For this same reason, the amplitude of the response was more difficult to manipulate under a normal transmembranal chloride gradient than under conditions of a reversed chloride gradient. Thus to further study the autoinhibitory postpotential responses in Retzius neurons, subsequent experiments were carried out using electrodes filled with KCl, which reversed and amplified the autoinhibitory postpotentials. This also allowed a clearer distinction between the autoinhibitory postpotential and the potassium-dependent fast hyperpolarizing postpotential characteristic of Retzius neurons (Stewart et al. 1989a).

To test whether the autoinhibitory postpotential was produced by 5-HT release, we blocked secretion produced by APs by perfusion with leech saline with magnesium substituted for calcium (Trueta et al. 2003). This manipulation completely and reversibly abolished the autoinhibitory postpotential (Fig. 2; n = 8), supporting the hypothesis that it had been produced by a substance released by the neurons in response to electrical stimulation. Further evidence that 5-HT release underlies the autoinhibitory postpotential came from neurons depleted from 5-HT by incubation for 7 days with reserpine. In these neurons the autoinhibitory postpotential was absent (Fig. 3A; n = 7), although it was produced normally by neurons that had been incubated for a similar time only with the vehicle (Fig. 3B, black arrow; n = 7). The characteristics of the APs, including the K⁺-dependent hyperpolarizing postpotential (Stewart et al. 1989a), were not affected in the neurons in which 5-HT release was abolished either by the presence of magnesium or by treatment with reserpine (gray arrows in Figs. 2A and 3A).

To rule out the possibility that the lack of autoinhibitory postpotential in reserpine-treated neurons was due to a reduced sensitivity of the neurons to 5-HT, we tested their responses to iontophoretic pulses of 5-HT. The reserpine-treated neurons displayed the characteristic responses to 5-HT (Fig. 3A, inset).

![Fig. 2.](http://jn.physiology.org/)

**Fig. 2.** The autoinhibitory postpotential of Retzius neurons is produced by a substance released by the neuron. Isolated Retzius neurons were stimulated with 10 current pulses of 10 ms at 30 Hz using an intracellular electrode filled with KCl. A: reversed autoinhibitory postpotential of a Retzius neuron bathed in a normal physiological solution containing 1.8 mM Ca²⁺ (black), a solution in which calcium was replaced by magnesium to block secretion (dark gray), and after recovery in normal saline solution (light gray). APs are truncated. The gray arrow indicates the potassium-dependent hyperpolarizing postpotential revealed when 5-HT release was blocked. B: mean amplitude of the autoinhibitory postpotential in response to trains from 8 neurons bathed in the solutions shown in A. The zero value is the resting potential of the neurons. The asterisk indicates significant differences with respect to the response in normal solution.
Moreover, synchronizing the 5-HT pulses with the depolarizing intracellular current pulses reproduced the autoinhibitory postpotential characteristic of untreated neurons (Fig. 3C; compare with Fig. 3B).

The results presented so far support that 5-HT released by Retzius neurons acts on autoreceptors to activate inhibitory chloride currents. As expected, iontophoretic application of 5-HT produced a reversible increase in the membrane conductance, as seen from the 20 ± 8% decrease in the input resistance of six neurons (Fig. 4). This decrease was consistent with that reported in Retzius neurons in the ganglion on bath application of 5-HT (Dierkes and Schlue 2005).

**Serotonin release reduces subsequent excitability**

The effect of the ongoing 5-HT release on the excitability of the releasing neuron was studied under physiological transmembrane chloride conditions (using electrodes filled with KAc) in neurons stimulated with trains at 1, 10, and 30 Hz, since the amount of synaptic 5-HT release in Retzius neurons depends on the firing frequency (Dietzel et al. 1986; Stewart et al. 1989b). Although the amplitude of the chloride currents would saturate as the membrane potentials equals $E_{Cl}$, the frequency-dependent summation of the conductance changes could produce an excitability decrease.

Depolarizing currents with amplitudes that consistently produced an AP in response to each pulse throughout a 1-Hz train failed to produce the whole sequence of APs in the train when the frequency was increased to 10 or 30 Hz (Fig. 5A). The failures to produce APs reached 23 ± 6% of the total number of pulses in trains at 10 Hz and 47 ± 2% in trains at 30 Hz ($n = 6$; Fig. 5B), suggesting that the cumulative effects of the 5-HT released on stimulation had decreased the excitability of the neurons. As expected, in Retzius neurons forming a synapse onto pressure-sensory (P) neurons in culture the presynaptic failures to produce APs during the train correlated with a...
Although Retzius neurons in the ganglion may reach physiological firing frequencies $\geq 50$ Hz (Velazquez-Ulloa et al. 2003), the failures of the cultured neurons at high stimulation frequencies might be due to the refractory period of the membrane after an impulse. However, within 7 min, a bath application of the serotonergic antagonist methysergide (140 $\mu$M) reversively eliminated the AP failures during successive 10-Hz trains (Fig. 6, A and B) in the 17 neurons tested. Washing out the drug with normal physiological solution restored the percentage of failures to their initial values ($17 \pm 3\%$; $n = 6$; Fig. 6, A, bottom trace and C).

The frequency dependence of the autoinhibition of excitability was also tested in six reserpine-treated neurons (Fig. 6, D–F). Four of these neurons stimulated at 10 Hz did not fail to produce APs during trains of 10 pulses (Fig. 6, D, middle trace and E). The remaining two neurons had 9 and 21% of failures, respectively. The average $5 \pm 4\%$ of failures was significantly lower than the $23 \pm 6\%$ of nontreated neurons (Fig. 6F). The AP failures were also absent during the trains in three other neurons stimulated at 5 Hz and two neurons stimulated at 1 Hz (Fig. 6E). Moreover, iontophoretic application of 5-HT in synchrony with the intracellular current pulses increased the percentage of failures in every reserpine-treated neuron (Fig. 6, D, bottom trace and E). In the six neurons stimulated at 10 Hz, the failure percentage was increased to $35 \pm 3\%$ (Fig. 6F).

**Modeling predicts a localized effect of autoinhibition at synaptic terminals**

Since on single impulses Retzius neurons release 5-HT from presynaptic endings (Dietzel et al. 1986), without any significant release from the soma (Trueta et al. 2003), the presence of autoinhibitory postpotentials shortly after single APs suggested that they had a synaptic origin. However, since isolated neurons in culture are electrotonically compact (Kuffler and Nicholls 1990), the autoinhibitory postpotentials produced in the release sites become superimposed on the active membrane properties localized in the primary axon and soma, obscuring the site of autoinhibition localization. On the other hand, the lack of precise identification of presynaptic release sites, their location within the neuropile, and the small diameters of the neurites make direct electrical recordings of presynaptic terminals in situ nearly impossible. Therefore as a way of studying the functional effect of 5-HT autoinhibition in presynaptic terminals formed by neuropilar branches we used the alternative strategy of computational model analysis.

We manipulated the parameters in the model until we reproduced the shape of the APs, the chloride-dependent autoinhibitory postpotentials (Fig. 7A), and the sequence of APs recorded in cultured Retzius neurons in response to a train of current pulses at 10 Hz (Fig. 7B; see parameters in figure legend). Then the same parameters were used to simulate autoinhibition occurring at the neuropilar terminals with and without branches (see METHODS). As shown in Fig. 7C, in neurites without branches the autoinhibitory postpotential produced by 5-HT release upon arrival of a single AP at the presynaptic endings shunted the terminals, thus reducing the amplitude of the subsequent APs while the conductance was active (Fig. 7C, dark gray trace). The local reduction in the amplitude of the APs was transient and reflected the kinetics of the chloride conductance (Fig. 7D), thus conferring a fre-
FIG. 6. AP failures in Retzius neurons are due to 5-HT autoinhibition. The electrical activity of Retzius neurons was recorded with KAc-filled electrodes upon stimulation with trains of current pulses at 10 Hz. A: representative responses of a neuron before (top trace), after 7 min of 140 μM methysergide application (middle trace), and after washing the drug (bottom trace). The scale applies to the 3 traces. B: percentage of AP failures before (control) and after different times with methysergide in 17 independent neurons. Each dot indicates data from one neuron. C: mean percentage of AP failures before and after 7 min of methysergide application, as well as after washing the drug. The control and methysergide values are the average from 17 neurons. Recovery values were obtained from 6 of these neurons. D–F: neurons treated with reserpine did not show AP failures. Retzius neurons were recorded with KAc electrodes and stimulated with a train of current pulses at different frequencies. D: recording of a control (nontreated) neuron (top trace) and of a neuron treated with reserpine (middle and bottom traces) stimulated at 10 Hz. The bottom trace shows the response of the same neuron upon iontophoretic 5-HT application. 5-HT pulses (arrows) were synchronized with intracellular current pulses. Scale in B applies to the 3 traces. E: percentage of AP failures in 11 neurons treated with reserpine and stimulated at different frequencies (●, 1 Hz; ●, 5 Hz; ■, 10 Hz) in the absence and presence of 5-HT (applied by iontophoresis). Each symbol represents a neuron. Lines bind the data of the same cell in the 2 conditions. F: mean percentage of AP failures in nontreated and reserpine-treated neurons stimulated at 10 Hz in the absence and presence of 5-HT iontophoresis. The asterisk indicates significant differences with respect to control.

quency dependence to autoinhibition. It is noteworthy that this local reduction occurred without changing the excitability at the AP initiation site in the primary axon (Fig. 7C, light gray trace). Adding three branches to the neurites (Vargas and De-Miguel, unpublished results), each with its own presynaptic terminal, further reduced the AP amplitude following the activation of the autoinhibitory current in the presynaptic endings (Fig. 7C, black trace). However, the reduction of the AP amplitude was gradual as it spread toward the presynaptic endings (not shown), instead of an abrupt reduction at the branching points, as would be expected if there were conduction block at this point (Cataldo and Brunelli 2005). This lack of conduction block at the branching points may be due to the insertion of active currents in the neurites, which were necessary for the propagation of APs to the presynaptic endings.

**DISCUSSION**

We studied autoinhibition induced by the ongoing 5-HT release during trains of impulses in individual leech Retzius neurons in culture. We showed that the activation of a chloride conductance by released 5-HT produces a rapid autoinhibition of excitability and reduces subsequent 5-HT release. Our results suggest that autoinhibition occurs locally at the release sites. We also predict that the autoinhibition in distant presynaptic terminals in situ reduces locally the amplitude of the incoming APs in a frequency-dependent manner.

It has long been known that autoreceptors in serotonergic neurons contribute to the regulation of their firing frequency (Blier et al. 1998; Göthert 1990; Munsch and Schlue 1993; Roberts et al. 2001; Sari 2004; Stanford et al. 2000; Verge et al. 1985). However, the autoinhibition exerted by 5-HT at its own release sites in individual neurons, and particularly on the immediately subsequent excitability and release, had not been previously studied. Our finding that 5-HT release by single APs induces a chloride-dependent hyperpolarization suggests that autoreceptors are located near or at the presynaptic release sites. Thus our results suggest that serotonergic autoinhibition occurs at the release sites in real time, as 5-HT is being released.

The obvious effect of a chloride current is the increase of the membrane potential on the entry of negative charges. However, because in physiological conditions $E_{Cl}$ is close to the resting potential, we do not expect a large hyperpolarization in response to the activation of a chloride conductance. Instead, opening of chloride channels sets a temporary equilibrium state, which in addition to the membrane shunt during the lifetime of the chloride conductance produces a powerful inhibition of excitability that in electrototically compact neurons decreased the firing rate by producing failures to initiate APs. In presynaptic terminals located in the neuropile, one could expect that the autoinhibitory postpotential could prevent the APs from invading the presynaptic terminals or even to
produce conduction block at branching points in the neurites. Conduction block has been shown to occur in mechanosensory neurons in the leech (Baccus et al. 2000; Cataldo and Brunelli 2005; Gu 1991), as well as in other preparations both in invertebrates (Grossman et al. 1979) and in vertebrates (Dyball et al. 1987; Luscher et al. 1983), and selectively reduces synaptic transmission from presynaptic terminals at certain neurite branches (Gu 1991). Our model simulations did not show conduction block or failures to initiate APs in neurites with or without branches. However, the amplitude of an AP following activation of the autoinhibitory current was substantially decreased to as low as 14% of the initial amplitude. This modulation of presynaptic APs has an effect similar to that of presynaptic inhibition of Ia afferents in the cat spinal cord, which is also dependent on the transient activation of a chloride conductance by γ-aminobutyric acid (for review, see Rudomin and Schmidt 1999), and a similar effect may be occurring in axons and presynaptic endings of other serotonergic neuron types.

As expected, the reduced excitability produced by 5-HT autoinhibition in culture is followed by a decrease of 5-HT release. Although spike failures were seen in the soma of the model when presynaptic terminals were placed in the primary axon (Fig. 7B), our model did not predict AP failures in the simulated neuropile presynaptic terminals. However, the great reduction in the AP amplitude can be expected to have effects similar to those of AP failures, since 5-HT release is strongly dependent on the amplitude of the presynaptic depolarization (Dietzel et al. 1986). In addition, the decrease in the firing frequency may affect the rates of synaptic facilitation and depression (Dietzel et al. 1986; Stewart et al. 1989b), thus producing variable release dynamics. Thus 5-HT regulates its own subsequent release through the regulation of the excitability of active presynaptic terminals. From the kinetics of the autoinhibitory conductance, we expect that the low tonic firing frequency of these neurons (Garcia-Perez et al. 2004) would not be affected by autoinhibition, whereas increasing the firing frequency may rapidly reduce the output of the neurons.

Retzius and P neurons express at least two types of chloride ionotropic 5-HT receptors (Lessmann and Dietzel 1991) thought to be responsible for the miniature potentials recorded from cultured synapses (Henderson et al. 1983). In addition, there are metabotropic serotonergic receptors coupled to chloride channels through the protein kinase A–dependent pathway, which produce slow synaptic responses in P cells (Sanchez-Armass et al. 1991). Although our results do not provide specific evidence for the receptor type producing autoinhibition, its slow kinetics suggests a metabotropic response. However, 5-HT diffusing out of the release site and acting on extrasynaptic receptors may also produce slow electrical responses, as happens in dopaminergic neurons (Sombers et al. 2009). Another possibility is the contribution of a calcium-dependent chloride conductance, since 5-HT also modulates calcium channels in vertebrate and invertebrate
neurons (Deterre et al. 1982; Kravitz et al. 1980; Paupardin-Tritsch et al. 1986; Pellmar and Carpenter 1980) and evokes calcium transients in the neuropilar branches of Retzius neurons (Beck et al. 2002; Dierkes and Schlue 2005). However, our results do not provide any evidence for this type of indirect modulation of chloride currents.

Localization of the autoinhibitory mechanism and functional relevance

Our records from Retzius neurons in the ganglion have failed to display significant autoinhibitory postpotentials after long trains of impulses (Cercós and Trueta, unpublished observations) produced by somatic microelectrode stimulation or by skin stimulation to activate the polysynaptic inputs from sensory neurons (Szczupak and Kristan Jr 1995; Velazquez-Ulloa et al. 2003). Consistently, our simulations of autoinhibitory responses in distant neuritic presynaptic terminals predicted the local shunting of the presynaptic release sites without somatic effects. Therefore the autoinhibition produced in culture may unveil physiological events occurring at the presynaptic endings, which are regenerated in the axonal stump in culture, whereas in the ganglion are located in distant neuropterms. The calcium transients induced by 5-HT in the neurites but not the soma of Retzius neurons (Beck et al. 2002; Dierkes and Schlue 2005) support this hypothesis by suggesting a selective distribution of certain 5-HT receptor types.

The small amplitude of the autoinhibitory postpotential and the proximity of the resting potential and $E_{Cl}$ makes it unlikely that autoinhibitory potentials initiated at distal release sites may reach the primary axon, where integration occurs. This compartmental distribution of autoinhibition suggests interesting physiological possibilities. Since Retzius neurons produce their APs in the primary axon, a localized reduction of excitability at the synaptic endings would affect only the hard-wired output of Retzius neurons, including the connections to P cells (Merz and Drapeau 1994) and to S cells (Crisp and Muller 2006), while leaving the integrative properties of the neurons and their firing frequency intact. This seems particularly relevant in the case of serotonergic neurons, since somatic or axonal secretion (Bunin and Wightman 1999; Trueta et al. 2003; for review see De-Migueel and Trueta 2005) would not be altered by the local presynaptic autoinhibition. Upon longer-lasting trains of electrical activity, another type of 5-HT-independent long-term autoinhibition reduces the neuronal firing in leech (Gocht and Heinrich 2007; Rose et al. 2006) and in lobster (Heinrich et al. 1999) serotonergic neurons. Although the 5-HT-dependent autoinhibition seems to be localized in the neurites, the 5-HT-independent long-term autoinhibition may affect predominantly the axosomatic compartments.

General relevance of autoinhibition to serotonergic systems

Serotonergic autoreceptors in vertebrates are not coupled to chloride channels, but instead they produce autoinhibition by the activation of potassium conductances (Aghajanian and Lakoski 1984; Lee et al. 2008; Nicoll et al. 1990). An interesting case is snail neurons, which have both chloride- and potassium-dependent responses to 5-HT (Gerschenfeld 1971). In mammals, 5-HT1A autoreceptors are localized in the somatodendritic territory of serotonergic neurons of the raphe nucleus (Blier et al. 1998; Verge et al. 1985) and increases in extracellular 5-HT decrease the somatic firing rate of these neurons (Fornal et al. 1994; Gobert et al. 1995; Rogawski and Aghajanian 1981; Trulson and Crisp 1986; Trulson and Frederickson 1987). By contrast, the same neurons insert 5-HT1B autoreceptors in their presynaptic terminals (Göthert 1990; Roberts et al. 2001; Sari 2004; Stanford et al. 2000). This suggests that serotonergic neurons may have a localized autoinhibition, functionally similar to that presented here. Thus an attractive speculation is that by distributing different autoinhibitory mechanisms on their membranes, serotonergic neurons locally regulate their electrical activity patterns and their different modes of 5-HT release.

In spite of their reduced numbers, serotonergic neurons in vertebrate and invertebrate serotonergic systems extensively innervate the CNS (for review see De-Migueel and Trueta 2005). This is consistent with their wide regulatory effects on many important functions (Simansky 1996; Yuan et al. 2005). The compartmentalization of the autoinhibition mechanism proposed here might allow the local regulation of 5-HT release in areas of the CNS controlling different functions. Autoinhibition might be an important factor regulating 5-HT release and synaptic plasticity over periods of sustained electrical activity. Although it may avoid synaptic depression and postsynaptic desensitization, it may at the same time reduce the behavioral consequences of an excessive 5-HT release.

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