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Serotoninergic modulation of sensory transmission to brainstem reticulospinal cells

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ABSTRACT

Sensory inputs are subjected to modulation by central neural networks involved in controlling movements. It has been shown that serotonin (5-HT) modulates sensory transmission. This study examines in lampreys the effects of 5-HT on sensory transmission to brainstem reticulospinal (RS) neurons and the distribution of 5-HT cells that innervate RS cells. Cells were recorded intracellularly in the *in vitro* isolated brainstem of larval lampreys. Trigeminal nerve stimulation elicited disynaptic excitatory responses in RS neurons, and bath application of 5-HT reduced the response amplitude with maximum effect at 10 μM. Local ejection of 5-HT either onto the RS cells or onto the relay cells decreased sensory-evoked EPSPs in RS cells. The monosynaptic EPSPs elicited from stimulation of the relay cells were also reduced by 5-HT. The reduction was maintained after blocking either NMDA or AMPA receptors. The local ejection of glutamate over, RS cell elicited excitatory responses that were only slightly depressed by 5-HT. In addition, 5-HT increased the threshold for eliciting sustained depolarizations in response to trigeminal nerve stimulation but did not prevent them. Combined 5-HT immunofluorescence with axonal tracing revealed that the 5-HT innervation of RS neurons of the middle rhombencephalic reticular nucleus (MRRN) comes mainly from neurons in the isthmic region, but also from neurons located in the pretectum and caudal rhombencephalon. Our results indicate that 5-HT modulates sensory transmission to lamprey brainstem RS cells.

INTRODUCTION

Sensory transmission plays a crucial role in modulating the activity of neural networks involved in the control of movements. In turn, the central networks exert powerful modulatory effect on the transmission of sensory inputs, thus gating the sensory inflow in relation to programmed neural activity. Several neurotransmitters systems have been shown to be involved in the modulation of sensory transmission. Among others, serotonin (5-HT) plays a very important role (Lopez-Garcia, 2006).

Several studies have shown that 5-HT modulates sensory transmission in the spinal cord. In mammals, sensory transmission to superficial and deep dorsal horn neurons is either depressed by 5-HT, (cat; Headley et al., 1978; Anwyl, 1990; and rat: Lopez-Garcia & King, 1996; Lopez-Garcia, 1998; Garraway & Hochman, 2001), or in a small proportion of cases potentiated by 5-HT (rat: El Yassir et al., 1988; Lopez-Garcia & King, 1996). Depression effects have also been reported in the spinal cord of lower vertebrates. In tadpoles (Sillar & Simmers 1994) and lampreys, 5-HT decreases the amplitude of excitatory post-synaptic potentials (EPSPs) recorded in large secondary sensory neurons (giant interneurons) in response to stimulation of primary afferents (El Manira et al., 1997). In frog motoneurons, 5-HT also depresses the EPSPs induced by dorsal root stimulation (Ovsepian & Vesselkin, 2006).

There is far less known about 5-HT effects on sensory transmission at the supraspinal level. Only one previous study in guinea pigs suggested that 5-HT depresses glutamate release from trigeminal primary afferents through presynaptic inhibition (Travagli & Williams, 1996). Thus, the aim of our study was to investigate the

modulatory effect of 5-HT on sensory inputs in brainstem lamprey preparation. In lampreys, brainstem reticulospinal (RS) cells constitute the main command neurons activating the spinal locomotor networks (Brodin et al., 1988; Grillner et al., 1988). They receive sensory inputs from several modalities to adapt locomotor activity to environmental conditions. For instance, strong sensory stimulation elicits sustained depolarizations with spiking activity in RS neurons that trigger swimming activity (Viana Di Prisco et al., 1997, 2000). Furthermore, the lamprey brainstem contains a rich 5-HT innervation (Steinbusch et al., 1981). There are 5-HT fibers surrounding the cell bodies of some of the large RS cells (Viana Di Prisco et al., 1994). Moreover, there is a rich 5-HT innervation within the trigeminal descending tract (Pierre et al., 1992), where the trigeminal sensory relay cells are located (Viana Di Prisco et al., 2005). The trigeminal inputs to RS neurons thus provide an excellent model to investigate 5-HT modulation of sensory transmission in the brainstem.

In the present study, we show that 5-HT depresses sensory transmission to RS cells through effects on both AMPA and NMDA receptor components of the excitatory responses. These 5-HT effects seem to be mostly mediated by presynaptic mechanisms and the 5-HT innervation of RS cells seems to arise primarily from neurons in the isthmic region.

MATERIALS AND METHODS

Animals

Physiological experiments were carried out on 72 larval sea lampreys (*Petromyzon marinus*) ranging from 11 to 16 cm in total body length and collected from streams entering Lake Champlain (QC, Canada). The anatomical experiments were carried out on 14 adult sea lampreys ranging from 14 to 55 cm in total body length. Spawning phase sea lampreys were collected from St. Mary's (ON, Canada) and Great Chazy (NY, USA) rivers, whereas the young adults were either collected from streams entering Lake Champlain (QC, Canada) or purchased from ACME Lamprey Co. (Harrison, ME, USA). All surgical procedures were carried out in accordance to the guidelines of the Canadian Council on Animal Care (CCAC) and were approved by University Animal Care and Use Committees at the Université de Montréal and Université du Québec à Montréal.

Brainstem preparation

The brainstem preparation was the same for both anatomical and electrophysiological experiments. Lampreys were anesthetized with tricaine methanesulphonate (MS 222, 100 mg/l, Sigma, Oakville, ON, Canada) and decapitated. Skin, muscles, cartilage, and viscera were removed. The brains were dissected out with their underlying cranium. A complete transection rostral to the mesencephalon and the brainstem preparation was then pinned down dorsal side up on sylgard at the bottom of a recording chamber. In all experiments, the preparation was continuously perfused with a

solution of oxygenated (with 100% O_2) cold Ringer's (8-10°C, pH 7.4) of the following composition (in mM): 130 NaCl, 2.1 KCl, 2.6 $CaCl_2$, 1.8 $MgCl_2$, 4 HEPES, 4 dextrose, and 1 $NaHCO_3$.

Electrophysiological recordings

Giant RS cells located in the MRRN were recorded intracellularly using conventional glass microelectrodes filled with 4 M potassium acetate (80-130 MΩ resistance). These neurons are referred to as Müller cells and can be identified visually under the dissecting microscope (Rovainen, 1967). The intracellular signals were amplified by an Axoclamp 2A amplifier (Axon Instruments Inc, Foster City, CA, USA) in bridge mode. The signals were then digitized using the Clampex 9.2 software (pCLAMP9 pack program; Axon Instruments Inc) through a Digidata 1322 interface (Axon Instruments Inc). Off-line analysis was carried out using the Clampfit 9.2 software (Axon Instruments Inc). RS neurons included in this study had a stable resting membrane potential lower than -65 mV (mean value: -77.8 ± 1.2 mV).

Stimulation

The ipsi- or contralateral trigeminal sensory root was electrically stimulated to elicit synaptic responses in RS cells. Stimulation was delivered with a glass-coated tungsten microelectrode (0.5-2 M , 10-25 µm tip exposure) using a Grass S88 stimulator (Grass Instrument, Quincy, MA, USA) connected to a stimulus isolation unit (SIU; Grass Instrument). Single shocks delivered every 30s (1-12 ms duration, 0.4-14 µA intensity) were systematically used to induce subthreshold responses. RS cell responses to

trigeminal stimulation are disynaptic and the relay cells have been shown to be located in the lateral parts of the brainstem, in the trigeminal descending tract (Viana Di Prisco et al., 2005). Electrical stimulation was also performed within the relay area to elicit monosynaptic EPSPs (1-7 ms duration, 1-10 µA intensity). To elicit suprathreshold responses, repeated trains of 2-4 pulses at 30 to 90 Hz were delivered every 0.2, 0.3 or 0.5 s. The repeated trains were applied for 1-5 s at generally larger intensities (2-20 µA). This stimulation paradigm elicited sustained depolarizations in RS cells that were often accompanied by action potentials. The interval between successive stimulation trials was kept to more than 3 min.

Drug application and solutions

5-HT (Sigma) was bath applied (10 µM) or locally ejected (1 mM) into the relay cell area or directly over the soma of RS cells. The local ejections were made by applying positive pressure pulses through glass micropipettes using a Picospritzer (General Valve Corporation, Fairfield, NJ, USA). The neutral dye Fast Green (Fisher Scientific, Nepean, ON, Canada) was added to the solution in the ejection pipette to monitor the size and the exact location of the local ejections made in the brainstem. This method was used previously by us and others to produce very localized actions of drugs (Viana Di Prisco et al., 2005; Paggett et al., 2004; Ryan et al., 2007). The recording chamber outflow was placed near the ejection site to avoid spreading of the ejected drugs onto surrounding brain areas. Ejections of Fast Green mixed with Ringer's did not produce any effect on RS cells.

In 4 experiments, Strychnine (Sigma; 5 μM) and Gabazine (Tocris Bioscience, Ellisville, MO; 10 μM), were bath applied to abolish inhibitory components in the synaptic responses elicited by trigeminal nerve stimulation. This was done to test possible potentiation of inhibitory inputs by 5-HT.

Glutamatergic antagonists, D,L-amino-5-phosphonopentanoic (AP-5, Sigma; 100 μM) and 6 cyano-7-nitroquinoxaline-2,3-dione (CNQX, Sigma, 20 μM) were dissolved in the Ringer's solution adjusted to pH 7.4 and bath applied. The two antagonists were washed in for 30 minutes, i.e well after their maximal effect (20 min after application; data not shown) was reached, before the effects of 5-HT were tested. The perfusion rate while bath applying the drugs was the same than under control conditions, approximately 2-3 ml/min.

The effect of 5-HT on glutamate-induced responses (D-Glutamic acid; Sigma, 5 mM) was tested in the presence of tetrodotoxin (TTX; Sigma; 1 μM). One micropipette containing glutamate mixed with Fast Green was directly positioned over the dendrites of the recorded RS cell. Another micropipette containing 1 mM 5-HT was placed over the soma of the same cell. Glutamate ejections (20-60 ms duration) were performed before and after a local application of 5-HT (lasting 30 s). The glutamate ejections were delivered every 30 s and five responses were measured and averaged.

Ca^{2+} Imaging

Calcium imaging was conducted in 14 experiments. The spinal cord was transversely sectioned at segmental level 2 or 3 and crystals of a calcium dye (Calcium Green-Dextran, 10,000 MW, Molecular Probes, Eugene, OR, USA) were applied to the

rostral stump of the cut spinal cord. The preparation was then put back into a dark chamber perfused with cold oxygenated Ringer's for a period ranging from 24-48 hrs. This consisted of the time needed for transporting the dye from the RS cut axons in the spinal cord to the cell bodies in the rhombencephalon. Images of labeled RS cell bodies were then observed on a Nikon Eclipse E600FN microscope (Nikon, Montréal, QC, Canada) equipped with a 20X water immersion objective and collected with an intensified Photometrics COOLSNAP HQ CCD camera (Photometrics; Trenton, NJ, USA). The images were acquired at a rate of 2-5 per second using a video image acquisition system. Intracellular recordings of a single RS neuron were systematically performed, as described above, in combination with calcium imaging.

Data Analysis

For each EPSPs illustrated, at least 5 traces were averaged in order to improve the signal to noise ratio. 5-HT induced a small hyperpolarization in the recorded RS cells (mean = 4.3 ± 0.8 mV, n = 38). However for better visual comparison of the synaptic responses, the electrophysiological traces in all figures were superimposed in control conditions and in the presence of 5-HT.

The effects of 5-HT, CNQX and AP-5 on the area of the EPSPs were measured. The area was measured from the beginning of the membrane potential depolarization after the stimulation artefact, until the membrane potential returned to resting values. The areas were expressed as a percentage of the control value. Throughout the paper, the data are indicated as the mean +/- SEM. All statistical analyses were performed on raw data using Origin software (OriginLab Corporation, Northampton, MA, USA). Student’s

paired t-tests were employed to compare drug effects between two groups. A one-way ANOVA followed by a post hoc Tukey analysis was used to compare means between more than two groups. A confidence level of $P<0.05$ was considered statistically significant.

Metafluor software (Meta Imaging Series 5.0) was used to analyze the imaging data. In each experiment (n=14), the level of fluorescence in 2-6 RS cell bodies (total of 57 RS cells) was measured and averaged for 4-5 trials before 5-HT application, 30 minutes after, and after washout. Comparisons of the effects of 5-HT were made between large RS cells (more than 80 µM) and small RS cells (less than 50 µM). The calcium responses were expressed as the changes in fluorescence $\Delta F/F$ (%) and mean areas are expressed in arbitrary units. These values were used for statistical analysis.

All figures were designed using CorelDraw 12 software (Corel Corp., Ottawa, ON, Canada).

Retrograde tracing and 5-HT immunofluorescence

Anatomical experiments were also carried out in 14 lampreys. Out of those, 9 were injected with dextran amines and 5 with biocytin. Texas Red-conjugated dextran amines (TRDA, Molecular Probes) or biocytin (Sigma) were injected in the MRRN as follows: First, the periventricular cell layer of the MRRN was disrupted with the tip of a glass micropipette in order to cut afferent axons. Immediately after the lesion was made, fine entomological needles with re-crystallized TRDA or biocytin on their tip were used to inject the lesioned area. The fibers of the medial longitudinal fasciculus that coursed just ventral to the large MRRN cells were spared in most cases, although there is a

possibility that some might have been cut with the glass micropipette. The brains were then transferred into a chamber perfused with cold Ringer's solution (8-10°C, 2-3 ml/min, total volume ~70 ml) overnight. The next morning, they were transferred into a phosphate buffered saline solution (PBS, 0.1 M, pH 7.4, 0.9% NaCl) containing 4% paraformaldehyde for 24 hrs and then transferred into a phosphate buffered 30% sucrose solution overnight. Cryostat sections of 25 µm thickness were collected on ColorFrost Plus slides (Fisher Scientific) and allowed to dry on a plate at 37°C overnight. The next steps were carried out at room temperature unless mentioned otherwise.

The next morning, all sections were rinsed three times for 10 min with PBS. The sections containing biocytin were then incubated for 1 h at room temperature in PBS containing streptavidin conjugated to Alexa Fluor 594 (Molecular Probes) diluted 1:200, and then rinsed three times 10 min in PBS. The biocytin- and the TRDA-containing sections were then all incubated in PBS containing 4% normal goat serum (Chemicon, Temecula, CA, USA) and 0.2% Triton X-100 (Fisher Scientific) for 1 hr. The latter solution was also used for antibody dilutions. The sections were then incubated overnight at 4°C in a rabbit anti-5-HT antibody (diluted 1:2000, Incstar, Stillwater, MN, USA) solution. After this, the sections were rinsed three times 10 min with PBS and then incubated in a solution containing a goat anti-rabbit antibody conjugated to Alexa Fluor 488 (Molecular Probes) at a dilution of 1:40 for 1 hr. They were then rinsed three times 10 min with PBS, quickly rinsed with dH_2O, and left to dry on a plate at 37°C for 15 min. They were then mounted with Vectashield DAPI (Vector, Burlington, ON, Canada). The sections were observed and photographed using an E600 epifluorescence microscope equipped with a DXM1200 digital camera (Nikon).

RESULTS

Effect of 5-HT on trigeminal evoked EPSPs

Low intensity single shock stimulation of a trigeminal nerve systematically evoked subthreshold EPSPs in RS neurons (Fig. 1A; see also Viana Di Prisco et al., 1995). Following bath application of 5-HT, a small hyperpolarization was observed in the recorded RS cells (mean = 4.3 ± 0.8 mV, n=38). On the other hand, the amplitude of the EPSPs elicited by trigeminal nerve stimulation was markedly decreased in all RS cells (Fig. 1B). The size of the depression varied with the concentration of 5-HT (0.6 μM = 77.8 ± 18.5% of control; 1 μM = 80.9 ± 15.4% of control; 5 μM = 45 ± 9.6% of control; 10 μM = 26.1 ± 5% of control; 30 μM = 28.4 ± 5.4% of control; Fig. 1D). A maximal depression was reached at 10 μM ($p < 0.001$, n=19). The concentration of 10 μM was thus used in the remaining experiments. The time course of the effects was also examined; about 20 min application period was necessary to achieve the maximal effect of 5-HT at 10 μM (Fig. 1E). All measurements were therefore made after 30 min bath application of the drugs.

The excitatory synaptic responses elicited by trigeminal inputs to RS neurons are known to be mediated by excitatory amino acids. However, it was also shown that trigeminal inputs consisted partly of glycinergic inhibitory inputs (Viana Di Prisco et al., 1995). The effect of 5-HT on the responses induced by stimulation of trigeminal nerve were studied in the presence of strychnine (5 μM) and Gabazine (10 μM), respectively blocking glycine and $GABA_A$ receptors. The amplitude of the EPSPs was significantly decreased by 5-HT application (19.8 ± 6.3 % of control, $p < 0.001$; n = 4 experiments;

Fig. 1C). The reduction of the EPSPs was similar to that seen without the glycine and $GABA_A$ receptor blockers. This suggests that the depression of sensory-evoked EPSPs by 5-HT is due to an action on excitatory synaptic transmission.

Localization of the effect of 5-HT

The effects of 5-HT could have been exerted on the connection between trigeminal primary fibers and relay cells, but also at the connection between relay cells and RS cells. Because of this, 5-HT (1 mM) was locally applied either on the recorded RS cells or in the relay cell area (Fig. 2A). The excitatory responses induced by stimulation of trigeminal nerve were reduced by ejections of 5-HT on RS cells (47.8 ± 6 % of control; $p < 0.001$; $n = 18$; Figs. $2B_1$, C_1). Local applications of 5-HT into the relay cell area, in the trigeminal descending tract, produced a similar decrease in the EPSPs (56.8 ± 9 % of control; $p < 0.001$; $n = 18$; Figs. $2B_2$, C_2).

The trigeminal relay area (Fig. 3A) was then stimulated to examine the effects of 5-HT on the synaptic transmission between the relay cells and RS cells. First, divalent cations (10.8 mM Ca^{2+}/7.2 mM Mg^{2+}) were added to the perfusion Ringer's in order to abolish polysynaptic transmission (Berry & Pentreath, 1976; Cazalets et al., 1995). The larger part of the response remained in the presence of the high divalent cations, suggesting that most of the response was monosynaptic (Fig. 3B). The amplitude of the excitatory responses elicited in RS cells by stimulation of the relay cell region was significantly decreased by 5-HT (30.6 ± 11.6 % of control; $p < 0.01$, $n = 7$; Figs. $3C_1$, $3C_2$). Taken together, these results suggest that sensory inputs to RS cells are depressed

by 5-HT and that the depression results from effects both at the connection between the primary afferents and relay cells and between the relay cells and RS cells.

Effect of 5-HT on glutamate receptor subtypes

The EPSPs induced in RS cells by trigeminal sensory stimulation were shown previously to consist of both AMPA and NMDA receptor components (Viana Di Prisco et al., 1995). Experiments were carried out to determine whether 5-HT acted on the two components. The NMDA receptor blocker, AP-5 (100 µM), slightly reduced the synaptic responses leaving the AMPA receptor component (Fig. $4A_1$), which was significantly reduced in the presence of 5-HT (14.3 ± 3.2 % of control; $p < 0.01$; $n = 13$; Fig. 4 A_2). Conversely, the AMPA receptor blocker, CNQX (20 µM), was added in 7 other experiments reducing significantly the EPSPs (Fig. $4B_1$). The remaining NMDA receptor-mediated component showed a marked decrease after 5-HT (11.1 ± 7 % of control; $p < 0.01$; $n = 7$; Fig. $4B_2$). These results suggest that 5-HT modulates monosynaptic EPSPs elicited in RS cells by stimulation of the trigeminal relay area, through effects on NMDA and AMPA receptor-mediated components.

The input resistance was measured in the recorded RS cell and there was no changes before and after 5-HT ($n = 5$) (10.8 ± 1.9 vs. 11 ± 1.8 M ; $p > 0.05$; data not illustrated). The absence of effect is in accord with previously published results (Viana Di Prisco et al., 1992). Presynaptic mechanisms were thus suspected, but to address a possible postsynaptic action, the effects of 5-HT were tested on the excitatory responses elicited by a direct local application of glutamate on the recorded RS cell. TTX (1 µM) was added to the Ringer's solution to eliminate indirect activation of the RS cell. The

depolarizations induced by glutamate application were affected by 5-HT, but only mildly (87.5 ± 9.7 % of control; $p < 0.05$; n = 7; Figs. 4C_1, C_2). The average hyperpolarization induced by 5-HT in the recorded RS cells under TTX was 4.5 ± 0.6 (n = 7).

Effect of 5-HT on sustained depolarizations elicited by sensory stimulation

Using stimulation trains and larger current intensities, the subthreshold EPSPs elicited in RS cells by trigeminal sensory stimulation turned into sustained depolarizations that considerably outlasted the stimulation trains (see also Viana Di Prisco et al., 1997, 2000). In 10 experiments, 5-HT was tested on these sustained depolarizations (Fig. 5). In the example shown, stimulation intensities of 4 µA and 5 µA elicited sustained depolarizations that lasted 5 s and 8.5 s, respectively (Figs. 5 A_2, A_3, control). After a bath application of 5-HT, the sustained depolarizations disappeared in RS cells (Figs. 5 A_2, A_3, 5-HT). However, they recovered by increasing the intensity or the duration of the stimulation trains (Figs. 5 A_3, B). These results suggest that 5-HT increases the threshold for eliciting the sustained depolarization rather than preventing their occurrence.

The sustained depolarizations elicited in lamprey RS cells are associated with an intracellular rise in Ca^{2+} (Viana Di Prisco et al., 1997, 2000). Because 5-HT reduced the size of the sustained depolarizations for the same stimulation strength, it was hypothesized that the drug would also reduce the rise in intracellular Ca^{2+} seen in RS cells in response to sensory stimulation. Calcium imaging was performed in 14 experiments and the effects of 5-HT were tested on the Ca^{2+} responses elicited in RS cells by trigeminal sensory stimulation (Fig. 6A). In 7 experiments, one RS neuron was

recorded intracellularly in conjunction with Ca^{2+} imaging (Figs. 6A, B; Cell 1 Recording). In response to suprathreshold sensory stimulation, RS neurons displayed sustained depolarizations accompanied by increases in relative fluorescence indicating an increase in intracellular Ca^{2+} concentration (Fig. 6B, control). The sustained depolarizing responses were decreased following bath application of 5-HT (Fig. 6B, 5-HT). The time course of the Ca^{2+} response was similar in the different RS cells (Figs. 6B, C, control). In 3 of the 14 experiments, 5-HT had no effects on the calcium responses (n = 8 RS cells). In the other 11 experiments (total of 49 RS cells), there was a significant decrease of the Ca^{2+} response (Fig. 6D, control = 207.1 ± 38.1; 5-HT = 42.4 ± 11.9; washout = 196.7 ± 43.2; $p < 0.001$ for 5-HT, 1-way ANOVA). The 5-HT effects were the same in all labeled cells in the MRRN, including large (Fig. 6E, n = 24; control = 246.1 ± 56.9; 5-HT = 48.6 ± 18.3; washout = 218.1 ± 61.6; see examples in Fig. 6C, cells 1, 2 and 3) as well as smaller (Fig. 6E, n = 25; control = 168.8 ± 42.9; 5-HT = 26.4 ± 11.9; washout = 167.7 ± 4.5; $p < 0.01$; see examples in Fig. 6C, cells 4, 5 and 6) RS cells. Recovery was obtained several minutes after washout of 5-HT.

Origin of the 5-HT innervation

The results above show prominent effects of 5-HT on the sensory transmission to RS cells, which raises the question of the origin of this 5-HT innervation. Experiments were thus carried out to investigate this matter using retrograde transport of biocytin (n = 5) or fluorescent dextran amines (n = 9), combined with 5-HT immunofluorescence (Fig. 7). Two of the biocytin-injected animals also had the rostral tier of their spinal cord examined for double-labeled neurons. Figure 7 illustrates the different double-labeled

neurons on photomicrographs (A-D) and shows their localization in the brain (top drawings a-e). A typical injection site is illustrated in Fig. 7d.

Double-labeled neurons were most consistently found in the ipsilateral isthmic 5-HT group (group II in Antri et al., 2006). They were present in 4 out of 5 animals injected with biocytin (total of 16 neurons) and in 2 out of 9 animals injected with dextran amines (total of 6 neurons). They were located in the periventricular cell layer, just ventral to the most rostral portion of the motor nucleus of the trigeminal nerve (V, Figs. 7c, C) and more rostrally, just ventral to the anterior rhombencephalic reticular nucleus (ARRN, Figs. 7b, B). The 5-HT isthmic cells were rostral to the MRRN at a distance of about 1 mm (approximately 700 µm in young and 1200 µm in spawning adults) from the rostrocaudal middle of the nucleus. The cell bodies were often tightly packed together with indiscernible individual shapes (Figs. 7B, C). Their level of 5-HT immunoreactivity appeared weaker than other 5-HT neurons in the isthmic group. The labeling of their nuclei with DAPI, which binds to DNA, facilitated the identification of individual cells (not shown). Exclusively present in biocytin-injected animals, rare double-labeled cells were found in the contralateral 5-HT nuclei of the pretectum, near the posterior commissure (group I in Antri et al., 2006; in 2 out of 5 animals, total of 2 neurons; Figs. 7a, A), and in the ipsilateral caudal rhombencephalic 5-HT nucleus (group III in Antri et al., 2006; in 1 out of 5 animals, total of 3 neurons; Figs. 7e, D). The double-labeled neurons in the pretectum (Fig. 7A) had small, ovoid periventricular cell bodies and the ones in the caudal rhombencephalic 5-HT group (Fig. 7D) had bipolar periventricular cell bodies and were part of the smaller 5-HT cells in that group. No double-labeled neurons were found in the spinal cord of the two biocytin-injected animals and only one neuron

was found in the hypothalamic 5-HT group from one biocytin-injected animal. This neuron had a round, bipolar cell body oriented in a medio-lateral fashion and giving rise to two primary dendrites. The apical dendrite appeared to be contacting the cerebro-spinal fluid in the ventricle. The anatomical results here show that the most of the 5-HT innervation to RS cells likely comes from cells in the isthmic region, although cells from the posterior commissure and caudal rhombencephalon regions may also contribute to some extent.

DISCUSSION

Results from this study show that 5-HT induces a depression of the trigeminal-evoked synaptic responses recorded in lamprey RS cells. The 5-HT effects occur both between trigeminal primary fibers and relay cells, and between relay cells and RS cells. They are exerted on both the NMDA and the AMPA receptor-mediated components of the EPSPs. Presynaptic mechanisms are most likely involved at least at the connection between relay cells and RS cells. The 5-HT effects also occur overall on the RS cell population of the MRRN as indicated by the Ca^{2+} imaging experiments. The 5-HT cells located in the isthmic region are likely to play a significant role in modulating sensory transmission to brainstem RS cells, at least on the second part of the trigeminal-RS pathway.

Serotoninergic modulation of the trigeminal sensory transmission

We found that 10 μM of 5-HT produced a maximal reduction of sensory transmission to RS cells. The same concentration was used by others in the brainstem (Viana Di Prisco et al., 1992) and spinal cord (Buchanan & Grillner, 1991) of lampreys. A dose-dependent effect of 5-HT was also reported on the synaptic transmission from RS neurons to spinal neurons (Schwartz et al., 2005; 2007), but maximal effects were obtained at much lower concentrations (i.e. 1 μM). In our experiments, higher 5-HT concentrations were possibly needed because the synapses are located deeper in the tissue as the brainstem tegmentum is thicker than the spinal cord. The trigeminal relay cells are located within the trigeminal descending tract (Viana Di Prisco et al., 2005), an area

located ventrally in the rhombencephalon, and the dendrites of RS cells in the MRRN occupy the entire tegmentum (Rovainen, 1967). The exact location of the synapses between the relay cells and RS cells has not been established yet.

Excitatory synaptic transmission from trigeminal afferents to RS cells is mediated by AMPA and NMDA receptors (Viana Di Prisco et al., 1995). Our results indicate that 5-HT depresses both the AMPA and NMDA receptor-mediated components of the EPSPs. In the lamprey spinal cord, 5-HT was shown to depress synaptic transmission and the effects have also been attributed to presynaptic mechanisms (Buchanan & Grillner, 1991; Shupliakov et al., 1995; El Manira et al., 1997; Blackmer et al., 2001). The subcellular mechanisms were recently investigated and 5-HT was shown to inhibit EPSPs by acting on G protein βγ-subunits (G-βγ) that modulate vesicle fusion properties (Gerachschenko et al., 2005; Photowala et al., 2006). The activation of the G-βγ subunits by 5-HT could then decrease the glutamate concentration in the synaptic cleft leading to a differential inhibition of synaptic NMDA and AMPA receptor-mediated currents (Schwartz et al., 2007). The authors have also reported a larger reduction of the AMPA than the NMDA receptor-mediated component. The doses of 5-HT were far less than those used in the present study. On the other hand, there was an additional reduction of the NMDA component at higher concentrations of 5-HT, in the range used in the present study (ca. 10 µM). In our study, 5-HT slightly reduced (≈ 13%) the depolarizing responses to a local application of glutamate over the RS cells. This small postsynaptic effect is unlikely sufficient to explain by itself the large depressive action of 5-HT on sensory transmission to RS cells. There were no effects on the input resistance of the recorded cells as previously reported (Viana Di Prisco et al., 1992). This suggests that the

5-HT effects reported in the present study are mostly exerted presynaptically at least in the second half of the trigeminal-RS pathway.

It is noteworthy that 5-HT induced a small hyperpolarization of about 4 mV on average in the recorded RS cells in the present study. A hyperpolarization was also observed in the presence of TTX. Similar results have been obtained in the past by Viana Di Prisco et al. (1992). This indicates that 5-HT does exert an effect at the postsynaptic level. However, whether the hyperpolarization can explain the depression of the excitatory synaptic responses observed under 5-HT is uncertain. A membrane potential hyperpolarization would result in an increase in the electrochemical driving force and thus the size of the EPSPs would be expected to increase rather than decrease. Moreover, as indicated above, 5-HT did not produce a significant change in input resistance of the RS cells and therefore a shunting effect is not likely to contribute significantly to the depressing effect of 5-HT on the sensory-evoked synaptic responses in RS cells. The postsynaptic hyperpolarization could depress an inward depolarizing current such as calcium current. This could lead to a decrease in calcium entry during the suprathreashold sustained depolarization, but this would need to be shown. On the other hand, we have previously shown that the sensory-evoked sustained depolarizations in RS cells are accompanied by large and sustained increases in intracellular calcium. Using a NMDA receptor blocker, we also showed that calcium entry through the NMDA receptors was likely to contribute markedly to the rise in intracellular calcium (Viana Di Prisco et al, 2000). Further experiments are needed to identify the subcellular mechanisms by which 5-HT depresses synaptic transmission to brainstem RS cells in lampreys.

Serotoninergic projections to the MRRN cells

In the lamprey brainstem, several regions contain 5-HT neuronal populations (Antri et al., 2006; Abalo et al., 2007). The present study shows that 5-HT innervation of MRRN cells seems to come from the brainstem and pretectum, and probably not from the hypothalamus and spinal cord. Despite the presence of numerous 5-HT neurons in diverse structures of the brain of lampreys, axonal tracing only labeled a few of these in our material. Biocytin was better at labeling 5-HT neurons than Texas Red-dextran amines. This could be due to the relatively smaller size of the biocytin molecule: This feature would allow biocytin to be picked up more easily by the very small-diameter 5-HT axons surrounding the MRRN cells, but also to diffuse in larger amount from the severed axons towards the cell bodies and label them more intensely. Biocytin has been shown to cross gap junctions between coupled neurons (Vaney, 1991). This could explain the larger number of double-labeled cells using biocytin, but evidence against this is that the general retrograde labeling pattern of neurons using Texas Red-dextran amines was the same as biocytin. Biocytin-labeled neurons seemed slightly more numerous than dextran-labeled neurons in general, and this simply reflected on the number of double-labeled neurons.

A reason why so few 5-HT neurons were found to project to RS cells could be that the latter possess large dendritic arborizations going deep in the tegmentum that could well be the target of a significant portion of the 5-HT inputs. We intentionally injected the tracers periventricularly to minimize the labeling of “en passant” axons in the medial longitudinal fasciculus for example, only aiming at RS cell bodies. Given these considerations, the number of regions containing 5-HT neurons projecting to RS cells

could have been underestimated. This was not a major concern in the present context, because we were only looking for candidate brain regions at the origin of the RS cell 5-HT innervation. Although much care was taken not to lesion "en passant" fibers, we cannot exclude that some have been labeled by the injections, such as some of the 5-HT neurons located in the isthmic region that are known to project to the spinal cord (Brodin et al., 1986). It will be important to study physiologically the different brain regions identified here to establish more precisely which ones provide the 5-HT innervation to RS cells.

Functional implication

The RS system plays a major role in controlling locomotor activity in vertebrates (for reviews, see Grillner et al., 1988; Mastuyama et al., 2004). It receives inputs from the periphery as well as from forebrain and brainstem locomotor centers. In lampreys, stimulation of the trigeminal nerve evokes escape swimming (McClellan, 1984; Cardin et al., 1999). The escape responses have been shown to result from sustained depolarization in RS neurons (Viana Di Prisco et al., 1997, 2000). We now show that 5-HT increases the threshold for eliciting sustained depolarization and can thus influence the total RS neuron output. Whether 5-HT modulation of RS responses also affects the overall sensory-evoked locomotor activity remains to be established. 5-HT could depress transmission in pathways involved in initiating swimming as well as sensory transmission during ongoing locomotor activity. The modulation is likely to be even more efficient with 5-HT effects not only exerted at the level of the connexion between relay cells and RS cells but

also at the first synaptic level in the Central Nervous System, between primary afferents and trigeminal relay cells.

Furthermore, RS cells are not functionally homogenous. It was shown that the function of different RS cells within one reticular nucleus for instance can differ considerably with respect to equilibrium and locomotor control (Zelenin et al., 2001; Deliagina et al., 2002). Although we now provide evidence that 5-HT is capable of depressing sensory transmission to all RS within one reticular nucleus, under natural conditions the selective activation of some populations of 5-HT cells may modulate transmission to specific RS cells reducing their inputs and perhaps activity with respect to other RS cells. We also provide evidence that 5-HT projections to RS cells of the MRRN arise from neurons located in the isthmic region as well as other brainstem areas. The selective activation of some 5-HT cells may provide a modulation at specific synapses originating from primary afferents to relay cells as well as from relay cells to RS cells. Therefore, through projections to different parts of the trigemino-reticular pathway, 5-HT neurons could exert both a general and more specific control of synaptic transmission.

Conclusions

A dense 5-HT innervation was described in the brainstem of lampreys in the past. We now show that RS cells of the MRRN are likely to be mostly innervated by 5-HT neurons in the isthmic region (group 2 of Antri et al., 2006). We also show that 5-HT exerts a powerful depression of transmission from trigeminal sensory inputs to RS cells. These effects could also play an important role in modulating inputs to RS cells in the

context of locomotion. Physiological experiments are underway to examine this in lampreys.

LEGENDS

Figure 1: 5-HT depresses the amplitude of subthreshold EPSPs induced by trigeminal nerve stimulation.

A: Schematic representation of the *in vitro* isolated brainstem preparation with a stimulation electrode positioned on one trigeminal nerve (V^{th} stim) and an intracellular microelectrode inserted in one large RS cell of the MRRN, identified visually. **B**: Synaptic responses elicited by a single shock stimulation (2 ms duration, 1.2 µA intensity) applied to the trigeminal nerve under control conditions (black line) and after bath application of 10 µM 5-HT (gray line). Each trace represents averages of 7 synaptic responses. **C:** Synaptic responses elicited by a single shock stimulation (8 ms duration, 2.6 µA intensity) applied to the trigeminal nerve under strychnine (5 µM) and Gabazine (10 µM) (black line), and after bath application of 10 µM 5-HT (gray line). Note that the membrane potential under each condition is indicated in parentheses for traces in B and C. **D**: Histogram of the mean area of the responses expressed as the % of control for different concentrations of 5-HT. Note that maximal depression of the EPSPs was achieved with 10 µM 5-HT (ns: non significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; paired t-test). **E**: Single stimuli of 3 µA were delivered every 30 s to examine the time course of the area of subthreshold EPSPs after 5-HT (10 µM) application. The areas were expressed in percentage of the control value. 5-HT effect shows a fast onset and requires about 20 min to reach maximum. Time zero represents the beginning of 5-HT perfusion.

Figure 2: The action of 5-HT is exerted both at the level of RS cells and that of the trigeminal relay cells.

A: Schematic representation of the *in vitro* isolated brainstem preparation showing the relevant circuitry. A stimulation electrode was positioned on one trigeminal nerve (V^{th} stim) and an intracellular microelectrode was inserted in one visually identified large RS cell in the MRRN. 5-HT (1 mM) was locally ejected either on RS cells (light gray encircled area) or into the relay cell area (dark gray encircled area). **B**: Synaptic responses elicited in response to a single shock (3 ms duration, 1.4 µA intensity, averages of 5 traces each) applied to the trigeminal nerve under control conditions (black line), after ejections of 5-HT (dark gray lines) on RS cells (**B_1**) or into the relay cell area (**B_2**) and after 1 hour washout (light gray lines). Note that the membrane potential under each condition is indicated in parentheses for traces in B_1 and B_2. **C**: Histogram of the mean area of the responses in control condition (black) and after 5-HT (gray) ejected either on RS cells (**C_1**) or into the relay cell area (**C_2**). *** $p < 0.001$; paired t-test.

Figure 3: Subthreshold EPSPs elicited in RS neurons by stimulation of the trigeminal relay area are inhibited by a 5-HT bath application.

A: Schematic representation of the *in vitro* isolated brainstem preparation with a stimulation electrode positioned into the relay cell area and an intracellular microelectrode inserted in one large RS cell in the MRRN. **B**: Monosynaptic inputs from relay neurons to RS cells revealed under control conditions (black line) and in the presence of 10.8 mM Ca^{2+}/7.2 mM Mg^{2+} (gray line; 50 min exposure). **C_1**: Synaptic responses elicited in response to a single shock (2.4 ms duration, 0.6 µA intensity,

averages of 7 traces each) applied into the relay cell area under control conditions (black line) and after 5-HT application (10 µM, gray line). Note that the membrane potential under each condition is indicated in parentheses for traces in B and C_1. **C_2**: Histogram of the mean area of the responses in control (black) and after 5-HT (gray). ** $p < 0.01$; paired t-test.

Figure 4: The effects of 5-HT on specific components of the excitatory responses evoked by sensory stimulation and on excitatory responses induced by local application of glutamate.

A_1: Synaptic responses elicited in response to a single shock (1.4 ms duration, 1 µA intensity, averages of 8 traces each) applied into the relay cell area under control conditions (black line), in the presence of AP-5 alone (100 µM; dark gray line) and in the presence of AP-5 (100 µM) with 5-HT (10 µM; light gray line). The inset shows the details of the boxed area in A_1. **A_2**: Histogram of the mean area of the responses in control (black), under AP-5 (dark gray) and under AP-5 with 5-HT (light gray). **B_1**: Synaptic responses elicited in response to a single shock (2.4 ms duration, 0.6 µA intensity, averages of 7 traces each) applied into the relay cell area under control conditions (black line), in the presence of CNQX alone (20 µM; dark gray line) and in the presence of CNQX (20 µM) with 5-HT (10µM; light gray line). The inset shows the details of the boxed area in B_1. **B_2**: Histogram depicting the mean area of the responses in control (black), under CNQX (dark gray) and under CNQX with 5-HT (light gray). **C_1:** Depolarizations elicited by local ejection of glutamate (5 mM, 30 ms duration, averages of 5 traces) on the recorded RS neuron. TTX (1µM) was perfused to block synaptic

transmission. Control traces are illustrated in black and those after local application of 5-HT (1mM) are illustrated in gray. C_2: Histogram of the mean area of the responses in control (black) and after local application of 5-HT (gray) showing a decrease of the glutamate responses. * $p < 0.05$, ** $p < 0.01$; paired t-test. Note that the membrane potential under each condition is indicated in parentheses for traces in A_1, B_1, and C_1.

Figure 5: Effects of 5-HT on the sustained depolarizations in RS cells.
A: Sustained depolarizations elicited in response to train stimulations (3 trains of 3 pulses at 30 Hz delivered every 300ms) applied to the trigeminal nerve under control conditions and after application of 5-HT. The trains of stimulation were delivered at different intensities: 3 (A_1), 4 (A_2) and 5 µA (A_3). At 4 or 5 µA (A_2 and A_3, control), the induced sustained depolarizations lasted 5 s and 8.5 s, respectively, whereas for the same stimulation intensities, the sustained depolarizations disappeared after 5-HT application (A_2 and A_3, 5-HT). A greater number of stimulation trains were needed to induce a sustained depolarization of similar size (A_3). Note that the membrane potential under each condition is indicated in parentheses for traces in A_1, A_2, and A_3. **B**: Relationship between the sustained depolarization areas induced by trigeminal nerve stimulation vs. the intensity of the stimulation under control and after 5-HT application.

Figure 6: 5-HT modulation of suprathreshold sustained depolarizations and concomitant intracellular calcium changes in RS neurons. **A**: Fluorescent image of MRRN neurons retrogradely filled with Calcium Green-dextrans. Six MRRN neurons were analysed in this example, including the intracellularly recorded cell (cell 1 recording, black circle).

Scale bar = 100 µm. **B**: Sustained depolarizations elicited in response to train stimulations (2 trains of 3 pulses at 90 Hz delivered every 200 ms) applied to the trigeminal nerve under control conditions, after application of 5-HT, and after washout. The electrophysiological responses were accompanied by increases in relative fluorescence (ΔF/F), indicating a rise in intracellular Ca^{2+} concentration. The sustained depolarization as well as the calcium response of the recorded MRRN cell were decreased in the presence of bath-applied 5-HT (10 µM). Note that the membrane potential under each condition is indicated in parentheses for traces in B. **C**: Calcium responses were measured in 5 other RS cells. The calcium responses were reduced in all analyzed RS neurons following 5-HT application. Note that the responses were restored after washout (rinse). **D:** Histogram of the mean area of calcium responses in 49 neurons from 11 experiments under control (black), 5-HT (gray) and after washout (white). E: Histogram depicting the mean area under the three experimental conditions, for large (more than 80 µm, n = 24) and small (less than 50 µm, n = 25) RS neurons. ns: non significant, ** $p < 0.01$, *** $p < 0.001$; One-way ANOVA.

Figure 7: Serotoninergic neurons from different brain regions send axons to the periventricular RS cells in the MRRN. Top drawings represent a dorsal view of the whole brain of a young adult lamprey (left), the black straight lines (a-e) correspond to the location of the cross sections illustrated on the right (a-e), and the tracer injection site is approximately located at the tip of the injecting pipette in red. A typical injection site is illustrated on a photomicrograph of a cross section in d. The photomicrographs labeled from A to D were taken from areas delineated by red squares on cross sections a, b, c and

e. Photomicrographs from the left column (biocytin) illustrate retrogradely labeled neurons, the ones from the central column (5-HT) illustrate the exact same frames with filter sets showing 5-HT-immunoreactivity, and the ones from the right column (biocytin + 5-HT) are a combination of the two previous photographs, with double-labeled neurons appearing in yellow-orange shades. A: Pretectum area showing a retrogradely labeled neuron with light immunoreactivity (A1-A3, arrows). B, C: Many neurons were retrogradely labeled in the isthmic region (B1-C1, arrows), some of them being also immunoreactive for 5-HT and often tightly packed together (B2, C2). Arrowheads point to darker profiles that represent nuclei of the 5-HT-positive neurons, as confirmed with DAPI labeling (not shown). D: The caudal rhombencephalic reticular formation with two retrogradely labeled neurons (D1; arrows). One neuron was also 5-HT-positive (D2,D3). Scale bar in D3 for all photomicrographs = 50 µm.

ABBREVIATIONS

5-HT:	Serotonin
AMPA:	Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ARRN:	Anterior rhombencephalic reticular nucleus
CNQX:	6-cyano-7-nitroquinoxaline-2,3-dione
AP-5:	D,L-amino-5-phosphonopentanoic acid
Mes:	Mesencephalon
mlf:	Medial longitudinal fasciculus
MRRN:	Middle rhombencephalic reticular nucleus
Mth:	Mauthner cell
NMDA:	*N*-methyl-D-aspartate
NMLF:	Nucleus of the medial longitudinal fasciculus
VIIIth:	Vestibular nerve
PRRN:	Posterior rhombencephalic reticular nucleus
Rh:	Rhombencephalon
RS:	Reticulospinal
SC:	Spinal cord
V:	Motor nucleus of the trigeminal nerve
VII :	Motor nucleus of the VIIth nerve
Vth:	Trigeminal nerve
X :	Motor nucleus of the Xth nerve

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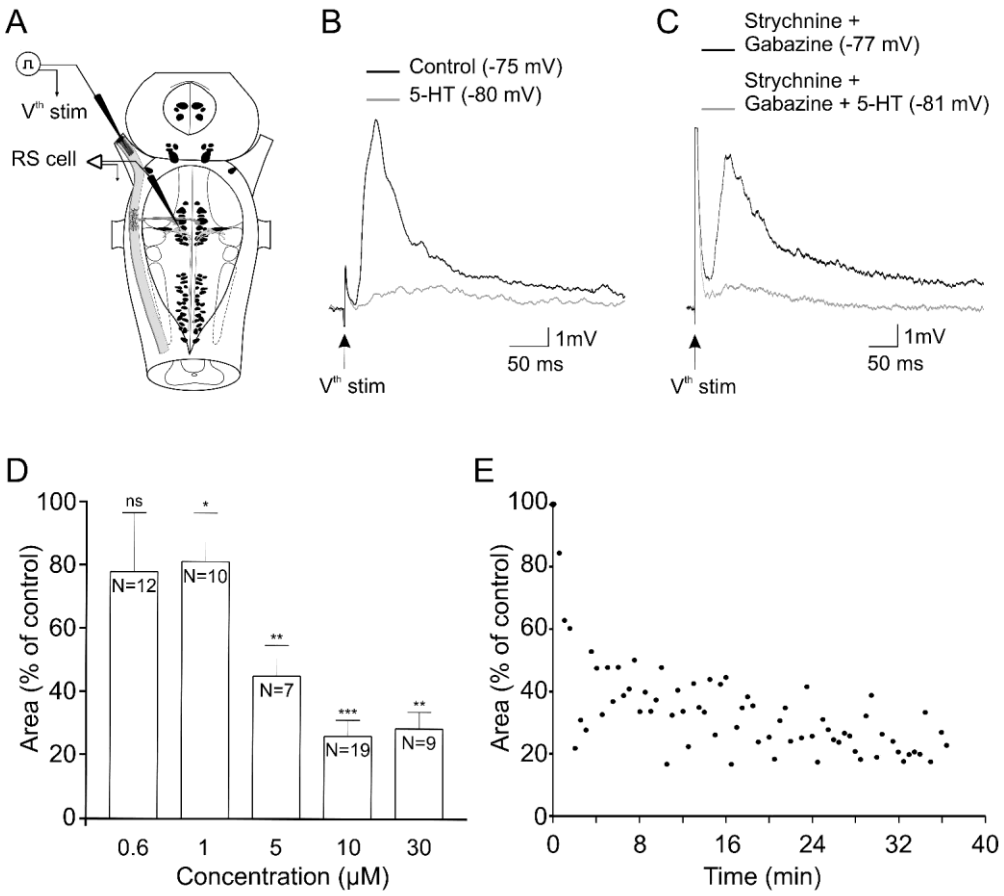

Figure 1: 5-HT depresses the amplitude of subthreshold EPSPs induced by trigeminal nerve stimulation. A: Schematic representation of the in vitro isolated brainstem preparation with a stimulation electrode positioned on one trigeminal nerve (Vth stim) and an intracellular microelectrode inserted in one large RS cell of the MRRN, identified visually. B: Synaptic responses elicited by a single shock stimulation (2 ms duration, 1.2 -A intensity) applied to the trigeminal nerve under control conditions (black line) and after bath application of 10 -M 5-HT (gray line). Each trace represents averages of 7 synaptic responses. C: Synaptic responses elicited by a single shock stimulation (8 ms duration, 2.6 -A intensity) applied to the trigeminal nerve under strychnine (5 μM) and Gabazine (10 μM) (black line), and after bath application of 10 -M 5-HT (gray line). Note that the membrane potential under each condition is indicated in parentheses for traces in B and C. D: Histogram of the mean area of the responses expressed as the % of control for different concentrations of 5-HT. Note that maximal depression of the EPSPs was achieved with 10 -M 5-HT (ns: non significant, * p < 0.05, ** p < 0.01, *** p < 0.001; paired t-test). E: Single stimuli of 3 -A were delivered every 30 s to examine the time course of the area of subthreshold EPSPs after 5-HT (10 μM) application. The areas were expressed in percentage of the control value. 5-HT effect shows a fast onset and requires about 20 min to reach maximum. Time zero represents the beginning of 5-HT perfusion.

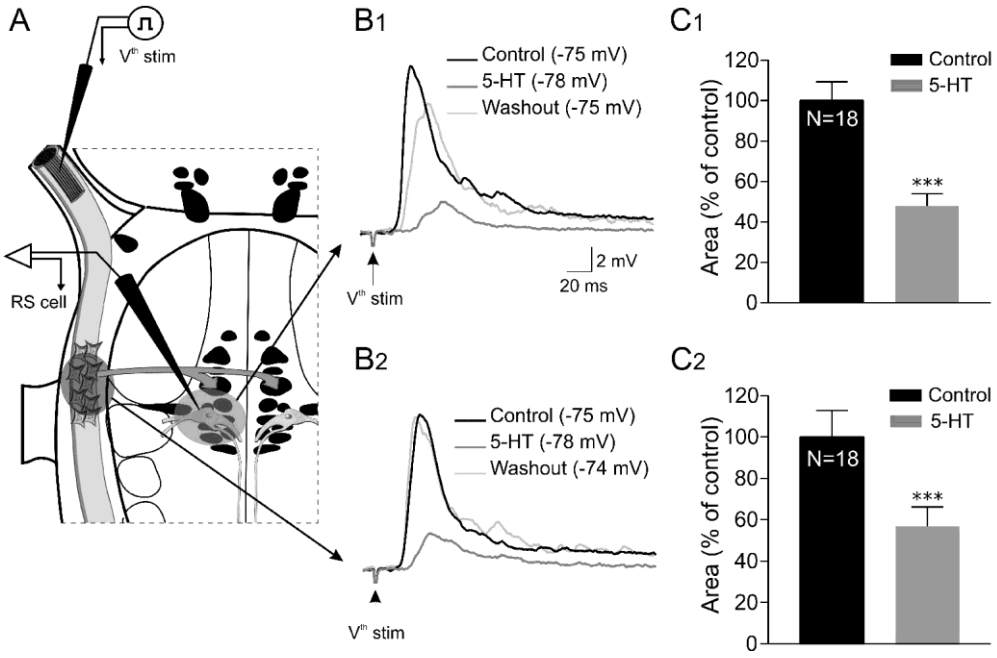

Figure 2: The action of 5-HT is exerted both at the level of RS cells and that of the trigeminal relay cells. A: Schematic representation of the in vitro isolated brainstem preparation showing the relevant circuitry. A stimulation electrode was positioned on one trigeminal nerve (Vth stim) and an intracellular microelectrode was inserted in one visually identified large RS cell in the MRRN. 5-HT (1 mM) was locally ejected either on RS cells (light gray encircled area) or into the relay cell area (dark gray encircled area). B: Synaptic responses elicited in response to a single shock (3 ms duration, 1.4 -A intensity, averages of 5 traces each) applied to the trigeminal nerve under control conditions (black line), after ejections of 5-HT (dark gray lines) on RS cells (B1) or into the relay cell area (B2) and after 1 hour washout (light gray lines). Note that the membrane potential under each condition is indicated in parentheses for traces in B1 and B2. C: Histogram of the mean area of the responses in control condition (black) and after 5-HT (gray) ejected either on RS cells (C1) or into the relay cell area (C2). *** $p < 0.001$; paired t-test.

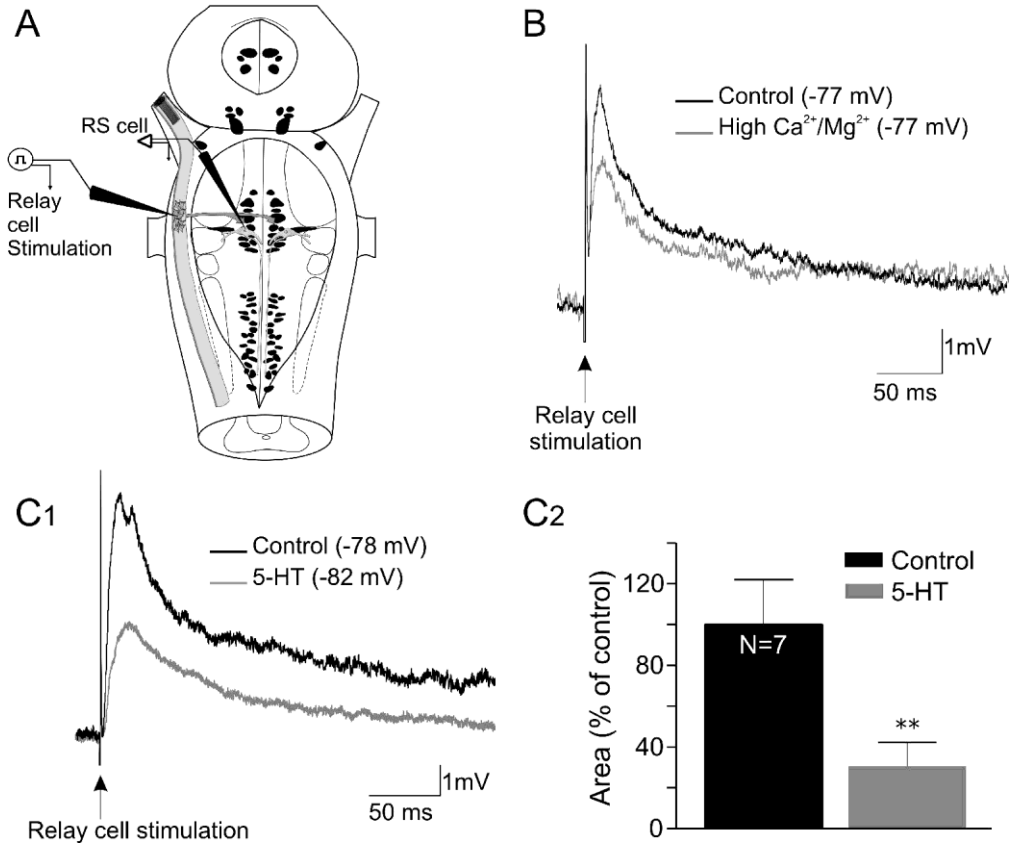

Figure 3: Subthreshold EPSPs elicited in RS neurons by stimulation of the trigeminal relay area are inhibited by a 5-HT bath application. A: Schematic representation of the in vitro isolated brainstem preparation with a stimulation electrode positioned into the relay cell area and an intracellular microelectrode inserted in one large RS cell in the MRRN. B: Monosynaptic inputs from relay neurons to RS cells revealed under control conditions (black line) and in the presence of 10.8 mM Ca2+/7.2 mM Mg2+ (gray line; 50 min exposure). C1: Synaptic responses elicited in response to a single shock (2.4 ms duration, 0.6 -A intensity, averages of 7 traces each) applied into the relay cell area under control conditions (black line) and after 5-HT application (10 -M, gray line). Note that the membrane potential under each condition is indicated in parentheses for traces in B and C1. C2: Histogram of the mean area of the responses in control (black) and after 5-HT (gray). ** p < 0.01; paired t-test.

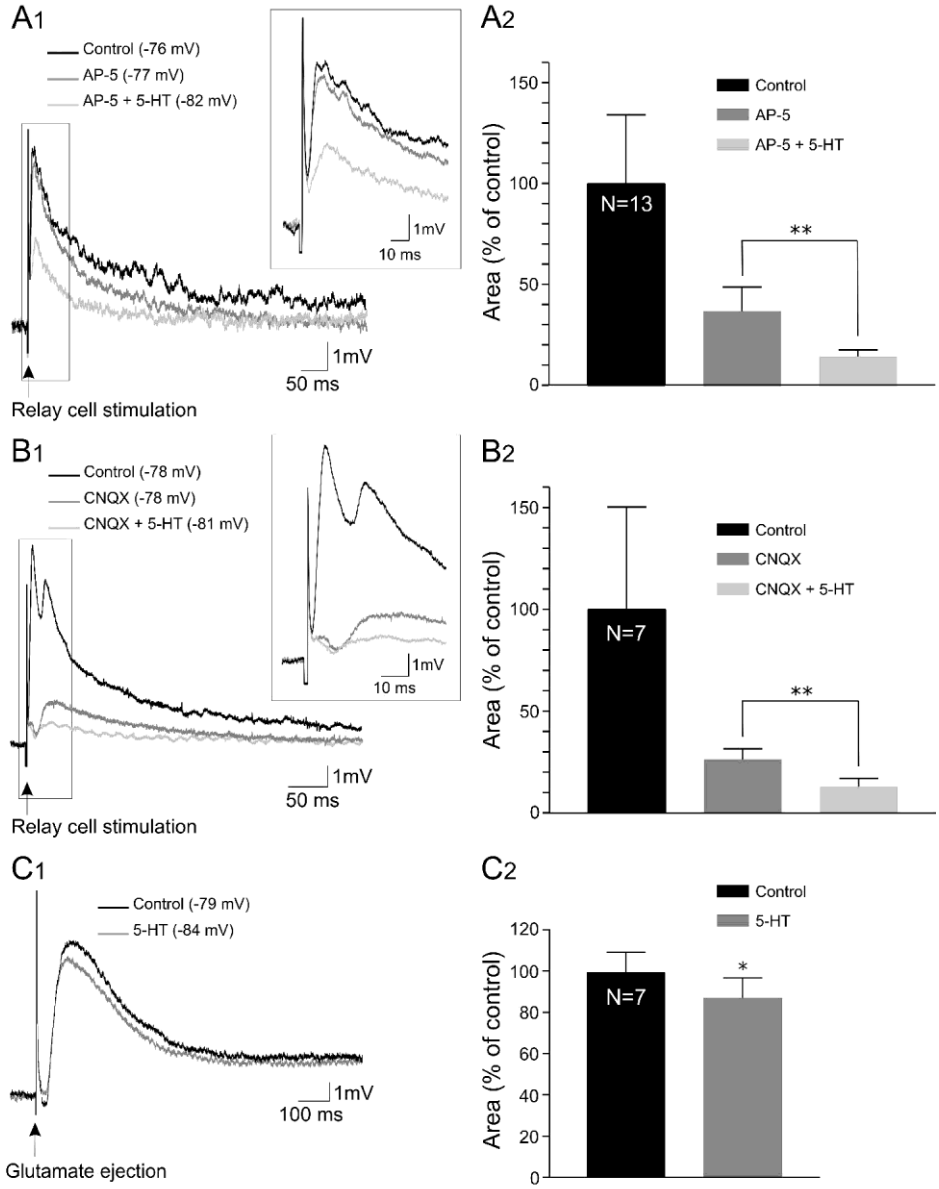

Figure 4: The effects of 5-HT on specific components of the excitatory responses evoked by sensory stimulation and on excitatory responses induced by local application of glutamate. A1: Synaptic responses elicited in response to a single shock (1.4 ms duration, 1 -A intensity, averages of 8 traces each) applied into the relay cell area under control conditions (black line), in the presence of AP-5 alone (100 -M; dark gray line) and in the presence of AP-5 (100 -M) with 5-HT (10 -M; light gray line). The inset shows the details of the boxed area in A1. A2: Histogram of the mean area of the responses in control (black), under AP-5 (dark gray) and under AP-5 with 5-HT (light gray). B1: Synaptic responses elicited in response to a single shock (2.4 ms duration, 0.6 -A intensity, averages of 7 traces each) applied into the relay cell area under control conditions (black line), in the presence of CNQX alone (20 -M; dark gray line) and in the presence of CNQX (20 -M) with 5-HT (10-M; light gray line). The inset shows the details of the boxed area

in B1. B2: Histogram depicting the mean area of the responses in control (black), under CNQX (dark gray) and under CNQX with 5-HT (light gray). C1: Depolarizations elicited by local ejection of glutamate (5 mM, 30 ms duration, averages of 5 traces) on the recorded RS neuron. TTX (1-M) was perfused to block synaptic transmission. Control traces are illustrated in black and those after local application of 5-HT (1mM) are illustrated in gray. C2: Histogram of the mean area of the responses in control (black) and after local application of 5-HT (gray) showing a decrease of the glutamate responses. * $p < 0.05$, ** $p < 0.01$; paired t-test. Note that the membrane potential under each condition is indicated in parentheses for traces in A1, B1, and C1.

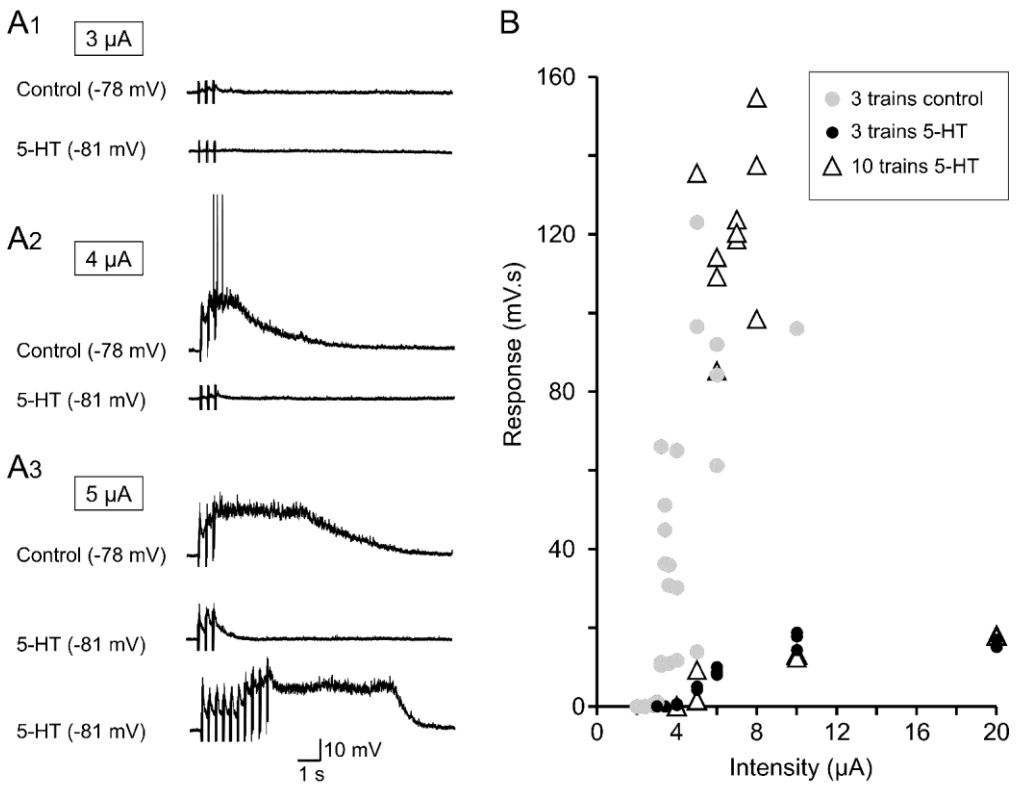

Figure 5: Effects of 5-HT on the sustained depolarizations in RS cells. A: Sustained depolarizations elicited in response to train stimulations (3 trains of 3 pulses at 30 Hz delivered every 300ms) applied to the trigeminal nerve under control conditions and after application of 5-HT. The trains of stimulation were delivered at different intensities: 3 (A1), 4 (A2) and 5 -A (A3). At 4 or 5 -A (A2 and A3, control), the induced sustained depolarizations lasted 5 s and 8.5 s, respectively, whereas for the same stimulation intensities, the sustained depolarizations disappeared after 5-HT application (A2 and A3, 5-HT). A greater number of stimulation trains were needed to induce a sustained depolarization of similar size (A3). Note that the membrane potential under each condition is indicated in parentheses for traces in A1, A2, and A3. B: Relationship between the sustained depolarization areas induced by trigeminal nerve stimulation vs. the intensity of the stimulation under control and after 5-HT application.

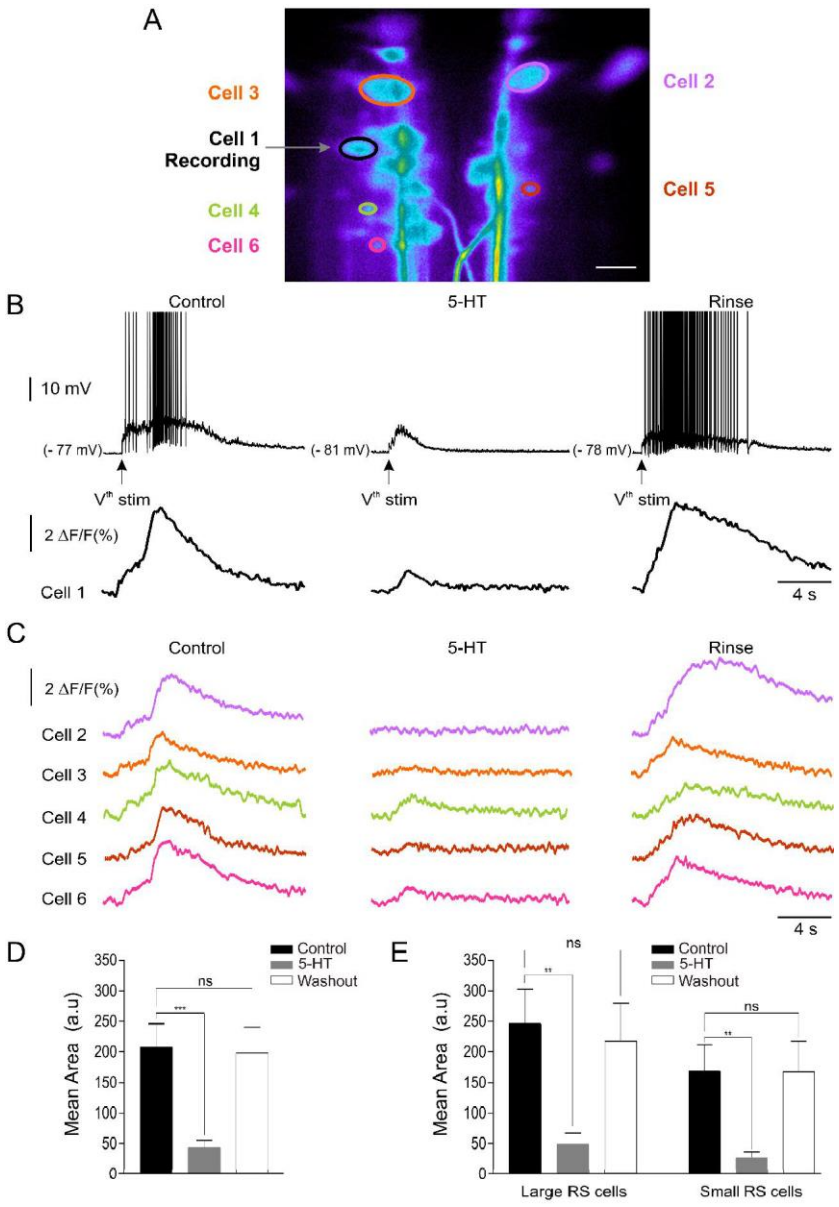

Figure 6: 5-HT modulation of suprathreshold sustained depolarizations and concomitant intracellular calcium changes in RS neurons. A: Fluorescent image of MRRN neurons retrogradely filled with Calcium Green-dextrans. Six MRRN neurons were analysed in this example, including the intracellularly recorded cell (cell 1 recording, black circle). Scale bar = 100 -m. B: Sustained depolarizations elicited in response to train stimulations (2 trains of 3 pulses at 90 Hz delivered every 200 ms) applied to the trigeminal nerve under control conditions, after application of 5-HT, and after washout. The electrophysiological responses were accompanied by increases in relative fluorescence (EF/F), indicating a rise in intracellular Ca2+ concentration. The sustained depolarization as well as the calcium response of the recorded MRRN cell were decreased in the presence of bath-applied 5-HT (10 -M). Note that the membrane potential under each condition is indicated in parentheses for traces in B. C: Calcium responses were measured in 5 other

RS cells. The calcium responses were reduced in all analyzed RS neurons following 5-HT application. Note that the responses were restored after washout (rinse). D: Histogram of the mean area of calcium responses in 49 neurons from 11 experiments under control (black), 5-HT (gray) and after washout (white). E: Histogram depicting the mean area under the three experimental conditions, for large (more than 80 -m, n = 24) and small (less than 50 -m, n = 25) RS neurons. ns: non significant, ** p < 0.01, *** p < 0.001; One-way ANOVA.

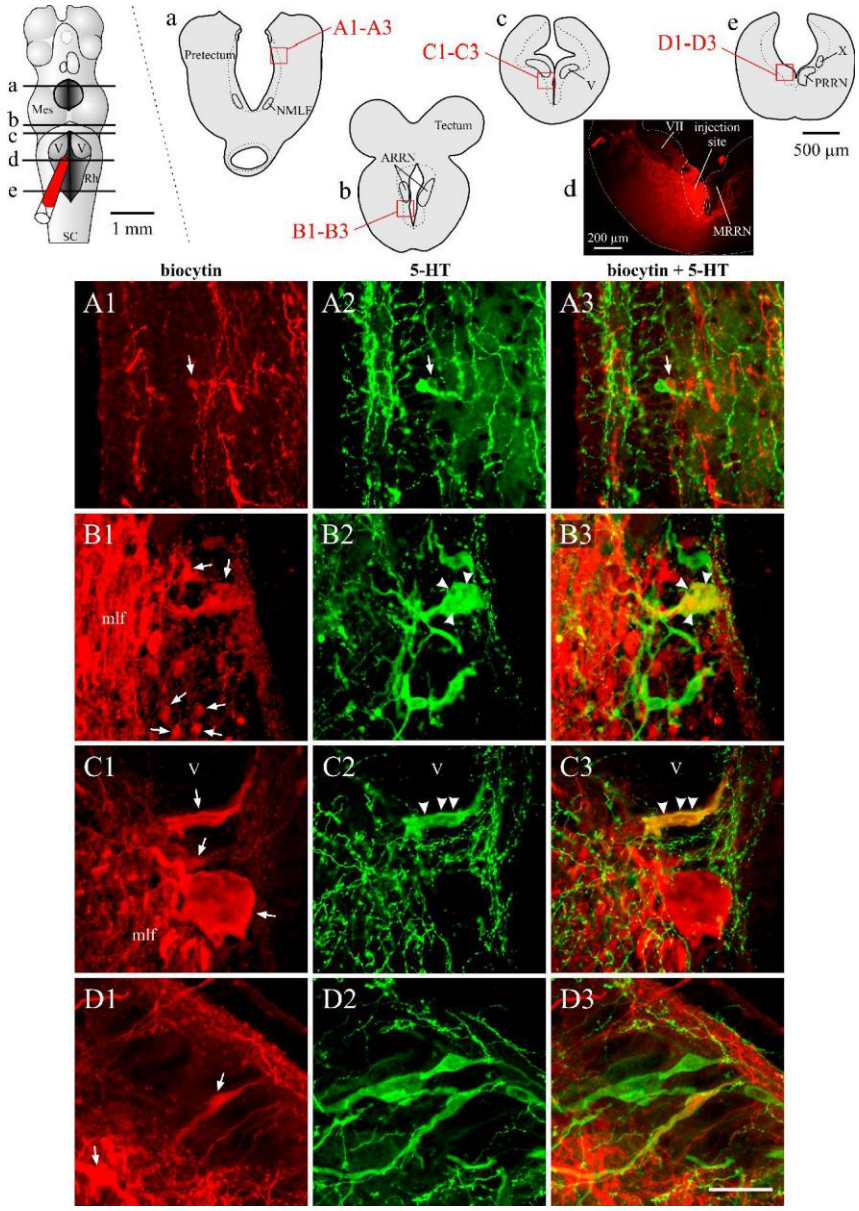

Figure 7: Serotoninergic neurons from different brain regions send axons to the periventricular RS cells in the MRRN. Top drawings represent a dorsal view of the whole brain of a young adult lamprey (left), the black straight lines (a-e) correspond to the location of the cross sections illustrated on the right (a-e), and the tracer injection site is approximately located at the tip of the injecting pipette in red. A typical injection site is illustrated on a photomicrograph of a cross section in d. The photomicrographs labeled from A to D were taken from areas delineated by red squares on cross sections a, b, c and e. Photomicrographs from the left column (biocytin) illustrate retrogradely labeled neurons, the ones from the central column (5-HT) illustrate the exact same frames with filter sets showing 5-HT-immunoreactivity, and the ones from the right column (biocytin + 5-HT) are a combination of the two previous photographs, with double-labeled neurons appearing in yellow-orange shades. A: Pretectum area showing a retrogradely labeled neuron with light immunoreactivity (A1-A3, arrows). B, C: Many neurons were

retrogradely labeled in the isthmic region (B1-C1, arrows), some of them being also immunoreactive for 5-HT and often tightly packed together (B2, C2). Arrowheads point to darker profiles that represent nuclei of the 5-HT-positive neurons, as confirmed with DAPI labeling (not shown). D: The caudal rhombencephalic reticular formation with two retrogradely labeled neurons (D1; arrows). One neuron was also 5-HT-positive (D2,D3). Scale bar in D3 for all photomicrographs = 50 8m.