Mechanisms of Serotonergic Facilitation of a Command Neuron

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Antonsen BL, Edwards DH. Mechanisms of serotonergic facilitation of a command neuron. J Neurophysiol 98: 3494–3504, 2007. First published September 26, 2007; doi:10.1152/jn.00331.2007. The lateral giant (LG) command neuron of crayfish responds to an attack directed at the abdomen by triggering a single highly stereotyped escape tail flip. Experimentally applied serotonin (5-hydroxy tryptamine, 5-HT) can increase or decrease LG’s excitability, depending on the concentration, rate, and duration of 5-HT application. Here we describe three physiological mechanisms that mediate serotonergic facilitation of LG. Two processes strengthen electrical coupling between the primary mechanosensory afferent neurons and LG: first, an early increase in the conductance of electrical synapses between primary afferent neurons and LG dendrites and second, an early increase in the membrane resistance of LG dendrites. The increased coupling facilitates LG’s synaptic response and it promotes recruitment of weakly excited afferent neurons to contribute to the response. Third, a delayed increase in the membrane resistance of proximal regions of LG increases the cell’s input resistance near the initial segment. Together these mechanisms contribute to serotonergic facilitation of LG’s response.

INTRODUCTION

The crayfish lateral giant (LG) neuron is an escape command neuron that responds to mechanosensory input elicited by an attack directed at the abdomen. The circuit mediates a critical mode of escape from predation and intraspecific aggression by producing a single highly stereotyped tail flip that propels the animal forward, up, and away from the attack. However, this escape directs the animal off the substrate and into the water column, where it may be visible to other predators, and therefore appropriate gain setting of the circuit is critical to the animal’s survival.

Weakly rectifying electrical synapses link terminals of primary mechanosensory afferent neurons (1°As) to the tips of the LG dendrites, and more strongly rectifying electrical synapses connect mechanosensory interneurons (MSIs) to LG at more proximal dendritic sites (Antonsen and Edwards 2003; Antonsen et al. 2005; Edwards et al. 1991; Zucker 1972). 1°As are also interconnected through a lateral excitatory network, which acts to selectively amplify phasic, directed stimuli by recruiting unstimulated 1°As that then add to LG and MSI excitation (Antonsen et al. 2005; Herberholz et al. 2002). These three distinct pathways leading to LG excitation provide many possible targets for modulation of circuit excitability.

In socially inexperienced crayfish, high serotonin (5-HT) concentrations (50 μM) applied quickly (fast high application) elicit transient inhibition, whereas low concentrations (5 μM) applied quickly (fast low application) elicit sustained facilitation. High concentrations applied slowly so that the bath concentration rises to 50 μM over 30 min (slow high application) elicit persistent facilitation. These results suggest that serotonergic modulation of LG occurs through at least two distinct, competing pathways (Teshiba et al. 2001). The pathways may themselves be plastic in response to social experience: the same slow high 5-HT application produces transient facilitation that washes out in socially dominant crayfish, whereas in social subordinates, it produces transient inhibition (Yeh et al. 1996, 1997). These data together suggest that serotonergic modulation of LG excitability is highly complex and may contain the capacity for differential activation under different contextual circumstances, although the physiological mechanisms leading to these different effects are largely unknown.

We describe here the physiological mechanisms that mediate serotonergic facilitation of LG’s responses. We found that 5-HT has facilitatory influences on the synapses between 1°As and LG and on LG’s distal and proximal membrane properties and that these influences differ in their sensitivities and time courses. Together, these effects act to amplify the ortho- and antidromic synaptic signaling that regulates LG’s excitability through the lateral excitatory network. This process of lateral excitation, in which the postsynaptic responses of distal dendrites antidromically recruit additional inputs from unstimulated presynaptic 1°A terminals, has provided a model of subcellular neuropilar computation. The results reported here show how these neuropil computations can be modulated by serotonin so as to affect entire circuits and the escape behavior of the animal.

METHODS

Animals and experimental setup

Crayfish (Procambarus clarkii) between 2.5 and 3.5 cm of both sexes were obtained from a commercial supplier (Atchafalaya Biological Supply, Raceland, LA). Animals were kept physically, visually, and chemically isolated in individual 1.5-l tanks for between 14 and 25 days before experiments. Anesthetization on ice for 20–30 min was followed by placing the animals dorsal side up in dishes containing physiological saline of the following composition (in mM) 202 NaCl, 5.37 KCl, 13.53 CaCl2, 2.6 MgCl2, 2.4 HEPES, pH 7.4. Low-Ca2+ saline was the same recipe except for (in mM) 1.35 CaCl2 and 14.7 MgCl2. The ventral nerve cords were exposed by removing the dorsal exoskeleton, viscera, and axial musculature. The ventral exoskeleton, head, and tailfan were left intact. The musculature of the uropods and telson was removed as completely as possible, taking care not to strain or damage the peripheral nerves of the terminal ganglion (A6) and to cut or damage as few 1°As as possible. The dorsal surface of the uropod protopod was removed to expose nerve 2.
(N2) for attachment of a stimulating suction electrode. The peripheral nerves of abdominal ganglia 1–5 were cut, and A6 was supported on a small piece of silicone elastomer (Sylgard, Dow Corning, Midland, MI). In some preparations, we separated the dendrites, initial segment, and soma of LG in A6 from the axon; we did this by ligating the ventral nerve cord just anterior to A6 with fine silk thread. This method did not appear to change the physiology of the LG, other than by increasing input resistance at the initial segment in the ligated, and therefore electrically smaller, cell, and thereby enhancing synaptic responses. Although it is possible to tease the LG axon out of the cord and ligate it directly, this requires removing the connective tissue sheath. Desheathing changes the dose responses of the cells to 5-HT, presumably because of better penetration of the bath solutions. The ligated preparations were left for 1 h before penetration with the electrodes and beginning the experiments. Electrodes in the axon were placed ∼0.5 mm anterior of the ligation, whereas electrodes in the initial segment were placed in the same location as in unligated preparations. Although the method of thread ligation we used does also ligate other axons, and therefore possibly removes descending input from LG, it was the best method available to us in these semi-intact preparations. We do not see any drift over time in the input resistance of either intact or ligated preparations (see Fig. 8 in RESULTS), and the sign and time course of the elicited 5-HT effects are the same in both.

The partially dissected preparation was then transferred to a Sylgard-lined dish with a volume of ~4 ml. Physiological saline at 19°C was circulated over the preparation at a rate of 0.35 ml/min. Serotonin was applied in 19°C physiological saline following one of two regimes: 1) slow high: 50 nM at 0.35 ml/min—tests using solutions of different conductivity found that this rate slowly raised concentration of the applied solution to ~95% of maximum after 30 min; 2) fast low: 5 μM at 7 ml/min, which raised the concentration to ~95% of maximum within 2 min. The wash was initiated 45 min after 5-HT onset using the same flow rates as used to apply 5-HT. Experiments were continued until 120 min after 5-HT onset. All experiments used for analysis are complete until at least the end of the washout period, but because all experiments involved at least one difficult-to-hold impalement in a small dendrite or similar, not all lasted the full 120 min due to electrodes becoming dislodged. Because we knew from experience that a stable preparation over a period of 15 min of stimulation (±<2% variation in responses) did not tend to drift much over 2 h, baseline measurements were normally done for 15 min before 5-HT was applied. Preparations that did not produce stable baseline responses were discarded. Controls were done over the 2-h 15-min period of the experiments.

Electrophysiology and analysis

Changes in synaptic potentials and input resistance were monitored in LG and sensory 1°As over the course of 5-HT application and subsequent washout. Extracellular shock to elicit a 1°A volley through A6 N2 was performed by attaching a bipolar suction electrode to the ventral surface of N2, far from the ganglion in the protopod (Fig. 1). Shocks were delivered from an AMPI (Jerusalem, Israel) Master-8-cp Stimulator passing through an A-M Systems (Carlsborg, WA) Model 2300 Stimulus Isolator and Model 1700 Differential AC Amplifier. Intracellular recordings and current injections were performed through an Axon Instruments (Sunnyvale, CA) Axoclamp 2B Amplifier, and data acquisition was through an Axon Instruments Digidata 1322A data-acquisition board and pClamp 9 software running on a Dell PC. Intracellular electrodes used for recording from LG dendrites or primary 1°As were filled with 3M KCl and had resistances between 30 and 60 MΩ. Electrodes for recording from LG initial segment or for injecting current into either the initial segment or 1°As had resistances between 20 and 30 MΩ. For locations of electrodes during particular experiments, see descriptions in RESULTS. For some experiments, it was necessary to confirm the location of the recording electrode by injecting intracellular tracers and subsequently imaging the preparations. The location of recorded 1°As was confirmed by iontophoretically injecting 3,000 MW anionic dextran-linked fluorescein (Invitrogen, Carlsbad, CA). To confirm LG dendrite recording sites, LG was filled with 3,000 MW dextran linked tetramethylrhodamine via picospritzing at the initial segment, whereas the recording site in the dendrites was iontophoretically injected with 3,000 MW anionic dextran linked Cascade Blue (Invitrogen). All tracers were the anionic, lysine fixable forms.

Data were analyzed using Prism software (Graphpad Software, San Diego, CA). Data were tested for normality, then one-way repeated-measures ANOVA were performed with Tukey-Kramer multiple comparisons posttests on data that passed the normality test. Friedman’s tests with Dunn posttests were used if the data did not pass the test for normality. To compare the relative changes between two recording sites (Fig. 3B and 5D), for each experiment, we took the mean of the baseline values (before 5-HT application), and set their ratio as 1. All subsequent data points were then normalized to this value, and statistics were performed as in the preceding text.

Imaging

After electrophysiology and filling of the recorded cells, preparations were transferred to freshly made 4% paraformaldehyde (Sigma, St Louis, MO) in crayfish saline, and left at 4°C overnight. Abdominal ganglia 5 and 6 were then removed from the tailfan, washed in distilled water for 5 min, then dehydrated to 100% ethanol in 10% steps of 10 min each. The ganglia were then transferred to a depression slide containing methyl salicylate (Sigma), and stored at −20°C until imaging. Filled cells were imaged in three colors on a Zeiss (Carl Zeiss, Thornwood, NY) LSM 510 confocal microscope using 20X Fluor air interface and 63X C-Apochromat water interface lenses. For all preparations, full images of all labeled structures were acquired using the ×20 objective and inter-slice intervals between 3 and 3.6 μm. Details were imaged with the ×63 objective and intervals of...
0.9–1.1 μm. All images were captured at 1,024 × 1,024 pixels and were stored with false colors based on the emission of the fluorophores. Projections were made from the stacks using the Zeiss software. Brightness of the images was adjusted if necessary to aid visualization of relevant structures. The image in Fig. 5 was derived from an image stack of seven slices taken with the ×63 objective, and brightness was uniformly increased 10% from the original image.

**RESULTS**

Facilitation induced by slow high 5-HT

A brief (0.3 ms) electrical stimulus delivered through a suction electrode to N2 of the terminal abdominal ganglion (A6) evoked a short-latency compound EPSP recorded in the LGIS (Fig. 2). The initial, fast rising component of the EPSP (α) resulted from the near synchronous input from 1°As in the stimulated nerve, whereas the later, larger wave of depolarization (β) resulted from MSI synaptic inputs (Figs. 1 and 2A). N2 shock sufficient to elicit an EPSP of 40–50% of LG’s firing threshold at the beginning of each experiment was used to monitor the effects of applied 5-HT. Each preparation was then shocked every 3 min until a stable baseline lasting ≥15 min was reached, when a slow superfusion of the preparation with 50 μM 5-HT was begun. The 5-HT concentration ([5-HT]) in the preparation bath increased slowly, building to 50% of full concentration over 30 min. This slow high 5-HT exposure elicited gradual, highly significant (repeated-measures ANOVA, \(F = 114.92\) for α, 180.31 for β; \(P < 0.0001; n = 6\)) increases in α and β EPSP amplitudes that were sustained after the 5-HT was washed out (Yeh et al. 1996, 1997) (Fig. 2).

Both the α and β EPSP amplitudes increased significantly from baseline within 3 min after 5-HT onset (Tukey-Kramer test, \(P < 0.01\); Fig. 2). The α EPSP amplitude increased from a mean response of 4.08 ± 1.40 to 5.21 ± 2.03 (SD) mV 30 min after 5-HT onset, a 27.7% increase. As outlined in METHODS, although all six preparations were maintained until at least the end of the wash, the change in SD apparent in the α EPSP at 78 min was due to the loss of the recording in one preparation at that time. An ∼28% enhancement was then maintained through the application period, which lasted 45 min, and the saline wash. The β EPSP amplitude continued to increase through the application period and into the saline wash, from a baseline of 6.26 ± 1.63 to 11.84 ± 3.49 mV 90 min after 5-HT onset (45 min after onset of the wash), after which the response amplitude remained at an ∼70% enhancement above baseline through the wash (Fig. 2).

Using paired intracellular recordings in the LG initial segment and in the dendrite contacting nerve 3 (N3) 1°As (Fig. 1), we found that the α EPSP elicited by N2 shock increased to a significantly greater degree (repeated-measures ANOVA, \(F = 3.10; P < 0.0001; n = 6\)) in the LG dendrite contacting N3 1°As than at the initial segment (Fig. 3). Twelve minutes after 5-HT onset, the α EPSP had increased 13.1% from 4.08 ± 1.40 to 4.39 ± 1.76 mV at the initial segment and 16.9% from 12.78 ± 1.35 to 14.95 ± 1.95 mV in the N3 dendrite, a significant (Tukey-Kramer test, \(P < 0.01; n = 6\)), 28.8% larger relative increase at the dendritic site (Fig. 3). After this, the differential between the two sites abated until the enhancement in the dendrites was ∼20% greater, and this was sustained through the wash. The time course of the 5-HT effect was the same in both locations, and in all six preparations, the greater enhancement in the N3 dendrite was detectable 3 min after 5-HT application onset (Fig. 3C). The changes in the graphs apparent at 78 min and again at 108 min are due to loss of the recordings in single preparations at those times.

Slow high exposure to 5-HT increases LG input resistance

LG’s input resistance, measured by two-electrode current clamp at the initial segment, increased significantly in response to slow high 5-HT application (repeated-measures ANOVA, \(F = 74.09; P < 0.0001; n = 6\); Fig. 4). Input resistance reached 15.3% above baseline 60 min after the onset of 5-HT application in intact nerve cords, from a mean of 0.121 ± 0.024 to 0.142 ± 0.027 MΩ (Tukey-Kramer test, \(P < 0.01\)). To gain insight into the location of the membrane changes leading to the input resistance increase, we ligated the LG using a silk thread tied around the nerve cord just anterior to A6, isolating the dendrites, soma, and initial segment from the rest of the cell. We then repeated the input resistance measurements both at the initial segment and in the axon (Fig. 4). Sixty minutes after 5-HT onset, input resistance at the initial segment in ligated preparations reached 71.9% greater than baseline, increasing from 0.576 ± 0.055 to 0.970 ± 0.081 MΩ (repeated-measures ANOVA, \(F = 293.78;\) Tukey-Kramer test, \(P < 0.01; n = 6\)). Input resistance in the axon between A5 and A6 decreased by 4.2% from 0.559 ± 0.043 to 0.541 ± 0.046 MΩ over the same time period (Fig. 4). This result suggests that increases in specific membrane resistance that produced the increased input resistance measured at the initial segment.
experiments and single electrode in the earlier study. Furthermore, LG grows with the crayfish (Edwards et al. 1994b), and even among crayfish of very similar size we have seen substantial differences in the size of LG dendrites and axon (unpublished data). This is presumably in large part responsible for the variation we saw in baseline input resistance. The influence of applied 5-HT on input resistance was consistent, however, regardless of baseline values.

Enhancements of $\alpha$ EPSP amplitude and LG input resistance follow different time courses in response to slowly applied 50 $\mu$M 5-HT. Input resistance did not increase significantly until 9 min after 5-HT onset (Tukey-Kramer test, $P < 0.01$; Fig. 4), whereas both $\alpha$ and $\beta$ EPSP amplitude increased significantly within 3 min (Tukey-Kramer test, $P < 0.01$; Fig. 2B). Furthermore, the $\alpha$ EPSP amplitude increased until 30 min after 5-HT onset, then plateaued, whereas input resistance continued to increase until 60 min after (Fig. 4), and the $\beta$ EPSP continued to increase until 90 min after (Fig. 2B).

**Slow high 5-HT enhances electrical coupling between 1°As and LG**

To determine whether increased coupling between the 1°As and LG might also contribute to the facilitatory effects of 5-HT on LG’s response to 1°A input, we measured the effects of 5-HT on antidromic coupling between LG and 1°As. N2 shock elicited an EPSP in LG dendrites; current produced by these EPSPs flowed antidromically through electrical synapses into unstimulated 1°As in N3 and produced an antidromic synaptic potential (ASP) there (Fig. 5A). In these experiments, the LG dendrite electrodes (LGdend3) were placed as close to the synapse as possible to limit the effect of changes in membrane resistance on measurements of junctional conductance. As a guide for proximity to the synapse, the 1°A was fired with occurred at or more distal to the initial segment, whereas little change in specific membrane resistance occurred in the axon. The membrane time constant, $\tau_m$, was calculated from the discharge after current pulse injections at the initial segment before and after 5-HT-induced facilitation developed. Before facilitation, $\tau_m$ was 9.9–12.3 ms, and after facilitation 14.1–28.4 ms. If the specific membrane capacitance is assumed to be constant and equal to 1$\mu$F/cm$^2$, the specific membrane resistance increased from 9.9–12.3 to 14.1–28.4 k$\Omega$-cm$^2$. The input resistance measurements obtained here are somewhat lower than previously published values, whereas $\tau_m$ is very similar (Edwards et al. 1994b). This is likely due in part to differences in technique: two-electrode current clamp was used for these
tetramethylrhodamine (red) through an electrode in the LG experiment, we filled LG with 3,000 MW dextran linked (LGDend3). Electrodes in the 1°A s were placed at the base of 30 mV) EPSPs at the dendrite recording site.

Baseline response of N3 dendrites (red) and 1°A ASPs at the dendrite recording site are indicated (*); in this case it is 18 μm from the dendritic recording site, and on one occasion the dendritic recording site was >150 μm from the 1°A contact; these preparations were not included in the analysis. In all other preparations, the dendritic recording site was between 18 and 145 μm from the 1°A contact. A confocal photomicrograph of one successful preparation is shown in Fig. 5B.

Both orthodromic EPSPs in a N3 dendrite branch of LG and antidromic synaptic potentials (ASPs) in an unstimulated presynaptic N3 1°A were significantly enhanced by slow high 5-HT application (repeated-measures ANOVA, $F = 103.37$ for dendrite, $98.60$ for 1°A; $P < 0.0001$; $n = 6$), with effects detectable within 3 min after 5-HT onset (Tukey-Kramer tests, $P < 0.01$; Fig. 5C). Thirty minutes after onset of 5-HT application the LG dendrite EPSPs had increased from $12.39 \pm 0.76$ to $17.40 \pm 1.63$ mV, a 40.1% increase, and 1°A ASPs had increased from $4.08 \pm 2.11$ to $6.01 \pm 3.45$ mV, a 46.0% increase (Fig. 5C). By taking the ratio of percent increase in ASP amplitude over the percent increase in dendritic EPSP amplitude, we see that ASP amplitude increased significantly more than did dendrite EPSP (repeated-measured ANOVA, $F = 10.97$; $P < 0.0001$) and that this was evident 6 min after 5-HT onset, when the 1°A ASP was enhanced $21.6 \pm 12.6\%$ more than the dendrite EPSP (Tukey-Kramer test, $P < 0.01$; Fig. 5D). This relative enhancement of the ASP then decreased to a sustained 10–15% after 15 min of 5-HT application. The sustained increase in the ratio of the ASP/EPSP amplitudes indicates that the electrical coupling between the 1°A s and LG was increased by 5-HT and that this increase was sustained after washout of the 5-HT. The very large relative increase in the 1°A ASP after 3 min may be misleading, as increases at both sites were very small.

Slow high 5-HT enhances lateral excitation among 1°As but does not directly influence intrinsic properties of 1°As

The mechanosensory 1°As within each sensory nerve projection that excite LG are also connected to other 1°As through ohmic electrical synapses to form localized lateral excitatory networks (Antonsen and Edwards 2003; Antonsen et al. 2005; Herberholz et al. 2002). Unstimulated 1°As in these networks receive converging synaptic inputs from stimulated 1°As and antidromic synaptic potentials from LG. Together, the inputs from neighboring stimulated 1°As and from LG can recruit unstimulated 1°As; their spiking responses, slightly delayed from the initial 1°A volley, then contribute to broadening the LG α EPSP and to excitation of MSIs that contribute to the LG β EPSP.

We found that 5-HT increased lateral excitation among 1°As. A suction electrode was used to stimulate one branch of N2 to provide input to the lateral excitatory network, and the
synaptic responses of an unstimulated $1^\circ$A in another branch of the same nerve were recorded by an intracellular microelectrode near its contact site with the LG dendrite. In all preparations, the unstimulated $1^\circ$A was recruited in response to an N2 stimulus that evoked a threshold synaptic potential of 8–16 mV at the recording site. The N2 shock was decreased until the potential in the $1^\circ$A was 50–70% of threshold, then 5-HT was applied slowly as described in the previous experiments. In an example from one of five preparations (Fig. 6A), a N2 shock evoked a 7.2-mV synaptic potential that was mediated by direct $1^\circ$A-to-$1^\circ$A connections and antidromic current flow from EPSPs in LG’s dendrites. Five minutes after 5-HT application, the synaptic potential increased to 9.1 mV, and within 10 min, this $1^\circ$A was recruited. In all preparations, the size of the synaptic potential increased to threshold within 15 min of the onset of 5-HT application. In all cases, recruitment was sustained through the 5-HT application and until $\geq$60 min after 5-HT washout.

5-HT application caused a slight, but significant and sustained decrease in $1^\circ$A input resistance (repeated-measures ANOVA, $F = 54.24; P < 0.0001; n = 6$). Six minutes after 5-HT application onset, multiple comparison tests revealed that input resistance had decreased significantly from $2.23 \pm 0.31$ to $2.21 \pm 0.34$ MΩ (Tukey-Kramer test, $P < 0.01$; Fig. 6B). The decrease in $1^\circ$A input resistance closely followed the time course of the increase in electrical coupling between $1^\circ$As and LG (Fig. 5D). Because input resistance measurements were taken in the $1^\circ$A axon close to its contact sites with LG dendrites, it is likely that the decrease in input resistance was caused at least in part by the increased coupling of the $1^\circ$As to LG.

To determine the effect of 5-HT on coupling between $1^\circ$As, pairs of coupled $1^\circ$As were impaled with microelectrodes, and one was fired with current injection $\geq 20\%$ above threshold to elicit an EPSP in the second. 5-HT application had no effect on $1^\circ$A-to-$1^\circ$A coupling strength (repeated-measures ANOVA, $F = 0.67; P = 0.8957; n = 6$) or the waveform of evoked spikes or EPSPs (Fig. 6, C and D). Between 3 and 6 min after 5-HT application, a small delay to the onset of the current-induced spike and the resulting EPSP in the second $1^\circ$A developed, possibly due to the decreased input resistance of the cell. These results indicate that the 5-HT-induced increase in lateral excitation resulted from an increase in coupling between $1^\circ$A and LG.

FIG. 6. Slow high 5-HT application enhances lateral excitation without directly influencing $1^\circ$A electrotonic properties. A: this N2 $1^\circ$A had a baseline PSP of 7.2 mV in response to N2 stimulation, which increased to 9.1 mV after 5 min of 5-HT application. Ten minutes after the onset of 5-HT application, the $1^\circ$A was induced to fire through the lateral excitatory network, and it continued to do so through 45 min of application and a 2-h wash (mean ± SD, $n = 6$). B: $1^\circ$A input resistance measured in the axon near the LG contact site was decreased slightly ($\geq 2.5\%$) but significantly ($P < 0.01$) by 5-HT. Conventions as for Fig. 2B (mean ± SD, $n = 6$). C: $1^\circ$A EPSPs produced in response to firing of a 2nd electrically coupled $1^\circ$A by intracellular current injection do not change in response to 5-HT application. D: spikes induced by depolarizing current injection in a $1^\circ$A have a slightly delayed onset after 5-HT application, possibly due to the slight input resistance decrease but otherwise appear unchanged. EPSPs in a 2nd, coupled, $1^\circ$A have the same onset delay during 5-HT application, but otherwise remain consistent. Insets: schematics of the experimental setups.

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Facilitation induced by fast low 5-HT

All the facilitatory effects described in the preceding text occurred in response to 50 μM 5-HT superfused onto the preparation slowly, so that 95% of maximum concentration was achieved in 30 min. Other modulatory effects occur with different patterns of 5-HT exposure (Teshiba et al. 2001). When the same concentration is applied quickly, so that maximum concentration is reached within 2 min (fast high exposure), LG is inhibited, and when a low concentration, 5 μM 5-HT, is applied quickly (fast low), LG is facilitated. The inhibition is due in part to a decrease in membrane resistance in the proximal LG dendrites (Glanzman and Krasne 1983; Vu et al. 1993), and we found that fast low-induced facilitation shares some but not all of the mechanisms described in the preceding text for the slow high facilitation.

First, we repeated the earlier experiments and also found that 5 μM 5-HT applied quickly (fast low exposure) significantly enhanced α and β EPSPs at the LG initial segment (repeated-measures ANOVA; \( F = 35.026, P < 0.0001 \) for α; \( F = 58.922, P < 0.0001 \) for β; Fig. 7A1). Thirty minutes after fast low 5-HT onset, the α EPSP had increased from 4.61 ± 0.48 to 5.72 ± 0.32 mV, a 24.1% increase, whereas the β EPSP had increased from 8.17 ± 0.22 to 11.25 ± 0.89 mV, a 37.7% increase. The time course of the fast low 5-HT elicited change in EPSP amplitude was very similar to that elicited by slow high 5-HT with significant changes in both α and β EPSP amplitudes detected 3 min after 5-HT onset (Tukey-Kramer tests, \( P < 0.01 \); Fig. 7A2). Second, the fast low exposure did not elicit a significant increase in input resistance measured by two-electrode current clamp at the initial segment of intact LGs (Fig. 7B), unlike the slow high exposure. In intact LGs, 5 μM 5-HT did not cause an input resistance increase from the baseline of 0.21 ± 0.02 MΩ (repeated-measures ANOVA, \( F = 0.6453; P = 0.9580; n = 6 \)). In ligated LGs, fast low 5-HT application did result in a significant input resistance increase (repeated-measures ANOVA, \( F = 3.01; P < 0.0001 \); \( n = 6 \); Fig. 7B1). However, the magnitude of the increase, from a baseline of 0.71 ± 0.03 to 0.75 ± 0.06 MΩ or an 5.6% increase 60 min after 5-HT onset, is only about 1/15th the enhancement seen with slow high 5-HT application (Fig. 7B2).

Control experiments performed using the same time period and stimulation parameters as used for the preceding 5-HT application experiments revealed that cellular responses are consistent over the time course used for these experiments (Fig. 8).

Discussion

The effects of single neuromodulators on synaptic, neuronal, and circuit responses are often complex. This complexity appears to result from the different sensitivities, sites of action, physiological mechanisms, and possible interactions of the second messengers that mediate these effects (reviewed in Katz 1999; Teshiba et al. 2001). Here we have begun to identify and localize the physiological mechanisms that mediate the facilitation in dendrites, but not from either an increase in 1°A excitability or an increase in coupling between 1°As.

**FIG. 7.** Fast low 5-HT superfusion enhances LG α and β EPSPs at the initial segment, but not input resistance. A1: LG EPSPs increase within 3 min after 5-HT onset (mean ± SD, \( n = 6 \)). A2: LG initial segment α and β EPSPs follow similar time courses in response to fast low 5-HT as they did for slow high 5-HT, although the magnitude of the β increase in fast low 5-HT is less than in slow high 5-HT. B1: fast low 5-HT application did not elicit an input resistance increase at the initial segment in intact LGs (LGIS; \( n = 6 \)), although in ligated LGs (LGISlig) there was a small increase (\( n = 6 \)). B2: input resistance increase in LGs ligated anterior to A6 in response to fast low 5-HT was a fraction of that seen in response to slow high 5-HT. Insets: schematics of the experimental setup; conventions as for Fig. 2B.
The present study describes the physiological mechanisms that mediate facilitation of LG’s response in socially isolated animals produced by the slow high and fast low exposures to 5-HT. Both slow high and fast low applications of 5-HT induced an increase in both the α and β EPSP at the LG initial segment (Figs. 2 and 7, respectively). The increase in the α EPSP in response to slow high 5-HT is produced in large part by an increase in the electrical coupling of 1°A terminals with LG distal dendrites (Fig. 5). The time course and magnitude of the α increase in response to fast low 5-HT is very similar to that for slow high, suggesting that the same mechanisms may be activated in response to both 5-HT application regimes. The slow high exposure, but not the fast low exposure, also evoked an increase in the input resistance of LG at the initial segment (Figs. 4 and 7, respectively). These mechanisms appear to account for the 5-HT-induced facilitation observed in LG’s responses to sensory nerve shock.

The increased electrical coupling between 1°As and LG was evident from the bidirectional increase in electrical transmembrane between the cells: both the orthodromic α EPSP in LG and the antidromic ASP in unstimulated 1°As were increased. We have identified two physiological processes that contribute to the increased electrical coupling, an early increase in the specific membrane resistance of LG’s distal dendrites, and an early increase in the conductance of the electrical synapses that connect 1°A terminals with LG dendrites. These processes were identified after considering the flow of synaptic current and the possible cellular changes that could produce increased coupling. Each major LG dendritic branch in A6 receives input from afferents in a different abdominal nerve (Antonsen and Edwards 2003). As a result, electrical stimulation of N2 excites afferents that synapse on the N2 dendritic branch but not on the N3 branch, so that the β EPSP recorded in the N3 dendritic branch was produced by spread of synaptic current from the N2 branch to the N3 branch (Fig. 1). As it spreads proximally to the initial segment, the α EPSP becomes greatly attenuated, showing that the initial segment and axon are the largest sinks for synaptic current. We found that the early effect of 5-HT was to increase the α EPSP in both the N3 dendritic branch and the initial segment, but that the relative (i.e., percentage) increase was greater in the dendrite than in the initial segment (Fig. 3). This result could occur if 5-HT initially had caused an increase in membrane resistance only in the LG dendrites. In this case, the synaptic current would evoke a larger α EPSP in the dendritic branch that received the input, and the EPSP would then spread with less attenuation across the dendritic tree. In areas of the cell where the increase in membrane resistance had not occurred, such as the initial segment and axon, the attenuation would be less affected and the increase in the α EPSP would be smaller. Other possible mechanisms, such as an increased synaptic current with no change in LG’s membrane resistance, or a membrane resistance increase restricted to the initial segment, would produce the same percentage increase in α EPSP amplitude throughout the cell, which we did not observe.

The conclusion that 5-HT also produced an early increase in electrical synaptic conductances between the 1°A and LG was reached through similar considerations. First, both orthodromic and antidromic electrical transmission increased. Second, the percentage increase in the ASP was greater than the increase in the EPSP that produced the ASP (Fig. 5, C and D). This could occur if the electrical conductance linking the dendrite and 1°A terminal increased, if the input resistance of the 1°A increased and so provided a smaller load for antidromic current from the LG dendrite, or if the membrane resistance of the afferent terminal and/or dendrite between the two recording sites increased. The second possibility did not occur: the input resistance of the 1°A terminals decreased (Fig. 6B). The third possibility, that the membrane resistance of the afferent termi-

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**Physiological mechanisms of facilitation**

The present study describes the physiological mechanisms that mediate facilitation of LG’s response in socially isolated animals produced by the slow high and fast low exposures to 5-HT. Both slow high and fast low applications of 5-HT induced an increase in both the α and β EPSP at the LG initial segment (Figs. 2 and 7, respectively). The increase in the α EPSP in response to slow high 5-HT is produced in large part by an increase in the electrical coupling of 1°A terminals with LG distal dendrites (Fig. 5). The time course and magnitude of...
nal increased, is also denied by this same result. However, our finding that the membrane resistance of the LG dendrites increased after 5-HT application is consistent with the last possibility. Despite this, the distal membrane resistance increase may have little additional effect on the ASP. The distance between the 1°A-LG synapse and the recording site in the N3 dendrite is <20 μm (Fig. 5B); even a very large increase in the membrane resistance of this distal length of dendrite is unlikely to produce the observed additional increase of 15–20% in the recorded 1°A ASP. This leaves only an increase in the electrical synaptic conductance as the mechanism able to account for the increase in the ASP amplitude. We conclude that in addition to causing an early increase in the membrane resistance of the LG dendrites, 5-HT also causes an increase in the conductance of the electrical synapses between the 1°As and the LG dendrites.

Aminergic modulation has previously been found to produce both increases and decreases in the conductance of electrical synapses. Dopamine increases the strength of electrical synapses made by VIII nerve afferents with the Mauthner cell in fish (Kumar and Faber 1999; Silva et al. 1995); like facilitation of the LG described here, this increases the sensitivity of the animal to stimuli that trigger escape. Dopamine also decreases electrical coupling between neurons in the vertebrate retina (Baldridge et al. 1998; Xia and Mills 2004) and hippocampus (Velazquez et al. 1997) through decreases in gap junctional conductance. Serotonin decreases axo-axonal electrical coupling in the S-cell network of the leech (Moss et al. 2005). In the case of the 1°A-LG synapses, it is not known whether serotonin increases electrical synaptic conductance by increasing the conductance of individual channels or by increasing the number of channels able to conduct electrical current.

The increase in antidromic coupling (Fig. 5, C and D) was accompanied by a decrease in the 1°A input resistance in the distal terminal (Fig. 6B), and no change in inter-1°A coupling (Fig. 6C) or in 1°A excitability (Fig. 6D). In the absence of other changes in the 1°A, the decreased input resistance of the distal terminal appears to result from the increase in conductance of the electrical synapses with LG dendrites.

In addition to the early increases in electrical synaptic resistance and membrane resistance of the LG dendrites, we also found that the slow high exposure, but not the fast low exposure, produced a delayed increase in the input resistance of LG at the initial segment (Fig. 4). This is likely the result of an increased membrane resistance in the more proximal part of the cell near the recording site.

The rise in LG input resistance at the initial segment became apparent 9 min after onset of the application 50 μM 5-HT when, extrapolating from our conductivity tests (see METHODS), the concentration in the bath would have reached ~25 μM. In contrast, the increase in both the dendritic input resistance and the junctional conductance were apparent within the first 3 min of 5-HT application, when the concentration would be no higher than ~8 μM. This minimum threefold difference in the apparent threshold concentrations suggests that the rise in synaptic coupling has a higher sensitivity to 5-HT than the rise in proximal input resistance. The sensitivity difference is not likely to result from a difference in the penetration of 5-HT into the tissue as the LG initial segment is at the dorsal surface of A6 while the dendrites are buried deep within the neuropil (Antonsen and Edwards 2003; Lee and Krasne 1993). Rather it is more likely to result from a difference in the 5-HT receptors (and their affinities for 5-HT) that mediate these effects.

**Synergies among mechanisms produce synaptic facilitation**

The serotonergic facilitation of the α EPSP described in the preceding text will enhance both α and β EPSPs in LG by both direct and indirect means. The α EPSP arises at the distal dendritic tips of the LG and is conducted passively to the initial segment where in adult crayfish it is too attenuated to reach LG threshold by itself (Edwards et al. 1994a, b; unpublished data). Instead, the LG is brought to threshold by the β EPSP, which is evoked 1–2 ms later by input from MSIs and sums with the declining phase of the α EPSP. Therefore whereas enhancement of the α EPSP will increase the overall synaptic response at the initial segment, enhancement of the β EPSP may be the more critical pathway for facilitation of the LG circuit and escape response.

The time course and magnitude of the α EPSP increase produced by fast low 5-HT application are similar to those produced by slow high application, and so we suggest that the same physiological mechanisms are activated. First, the early increase in the electrical conductance of synapses between 1°As and LG will increase the synaptic current flow evoked by each presynaptic 1°A spike, and so evoke a larger α EPSP in LG (Fig. 9, 1). The increase in membrane resistance of the LG dendrites will enhance the spread of the synaptic current laterally into neighboring LG dendrites and proximally into the initial segment. The larger EPSPs produced in LG dendrites...
will then evoke correspondingly larger ASPs in unstimulated 1°As (Fig. 9, 2). Those ASPs will be additionally increased by the increased synaptic conductance, which permits the LG EPSP to drive more current antidromically into the afferent terminals. The larger ASPs will assist the inputs from coupled afferents to recruit unstimulated 1°As. The spikes evoked by the recruited 1°As will provide additional excitation of LG through their own electrical synapses and will help excite MSIs through nicotinic cholinergic synapses (Antonsen and Edwards 2003; Herberholz et al. 2002; Miller et al. 1992) (Fig. 9, 3). The MSIs synapse on LG through rectifying electrical synapses on the proximal dendrite or initial segment. Their additional excitation in the presence of 5-HT will increase the synaptic current that underlies the β EPSP in LG (Fig. 9, 4). The effect of the larger MSI input will then be amplified by the delayed increase in LG’s input resistance at the initial segment to create a much larger β EPSP (Fig. 9, 5); this will, in turn, sum with the larger α EPSP and bring LG closer to threshold Fig. 9, 6).

Multiple modulatory effects of 5-HT on LG

The mechanisms of facilitation described here can be considered together with the mechanisms of inhibition evoked by the fast high application of 5-HT (Teshiba et al. 2001) to obtain an overview of serotonergic modulation of LG’s response (Table 1). Whereas the slow high and fast low applications evoked an increase in LG’s dendritic membrane resistance to produce facilitation, the fast high application produced a decrease in LG’s dendritic membrane resistance that led to inhibition (Vu et al. 1993). It is not yet known whether the fast high inhibition is also mediated by a decrease in electrical coupling between 1°As and LG. Slow high application (but not fast low) produced a delayed increase in the input resistance at the initial segment that contributed to facilitation, whereas the fast high application produced no apparent change in that input resistance to contribute to inhibition (Vu et al. 1993).

Cyclic AMP (cAMP) has been shown to be sufficient to produce slow high facilitation (Araki et al. 2005; Edwards et al. 2002). The slow high facilitation appears to be mediated by distinct and competing second messenger mechanisms (Teshiba et al. 2001). Other second messengers have not yet been identified, but it is likely that one and perhaps two others are involved, given the different time courses, sites of action, and sensitivities to 5-HT of the modulatory effects and their physiological mechanisms. Two crustacean G-protein-coupled 5-HT receptors have been identified: 5-HT$_{1A}$, which down-regulates cAMP, and 5-HT$_{2B}$, which activates PLC and IP3 (Spitzer et al. 2004). Whether these or other unknown receptors mediate the facilitatory and inhibitory effects of 5-HT on LG’s response remains to be determined.

<table>
<thead>
<tr>
<th>5-HT Application/ Sites and Physiological Parameters</th>
<th>Facilitation</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1°ALG synaptic conductance</td>
<td>Up</td>
<td>?</td>
</tr>
<tr>
<td>Distal LG dendrite resistance</td>
<td>Up</td>
<td>Down</td>
</tr>
<tr>
<td>LG initial segment resistance</td>
<td>Up (delayed)</td>
<td>?</td>
</tr>
<tr>
<td>cAMP</td>
<td>Up</td>
<td>?</td>
</tr>
</tbody>
</table>

5-HT, serotonin; LG, lateral giant; 1°AS, primary sensory afferent neurons.

The different sensitivities, time courses, and locations of the modulatory effects and mechanisms raise the question of what sources and release patterns of 5-HT might selectively activate these effects in the intact animal. 5-HT is both a hormone and a neurotransmitter in decapod crustaceans (Beltz and Kravitz 2002; Tierney et al. 2004). 5-HT is released into the blood from the pericardial organs and from secretory neurons in the abdominal and thoracic nerve roots (Beltz and Kravitz 1983; Christie et al. 1995; Spitzer et al. 2005). This release pattern might produce a low or slowly rising concentration of 5-HT in the blood that activates the slow high or fast low mechanisms of facilitation. 5-HT is also released onto neuronal targets in the CNS by 5-HT containing neurons. One pair of those neurons projects along the ventral nerve cord of crayfish, immediately ventral to the LG axons (Yeh et al. 1997). Each neuron has terminal varicosities on the initial segment of the LG axon in each abdominal segment that may provide rapidly rising, high concentrations of 5-HT at adjacent receptors on LG. At present, no physiological tests of the modulatory effect of this cell on LG have been made. Apart from this cell, no other 5-HT immunoreactive processes appear to lie immediately apposed to the axon or dendritic processes of LG, although serotonergic processes in the neuropile of the terminal abdominal ganglion surround the dendritic terminals of LG (Edwards et al. 2002). It appears likely, therefore that the LG is modulated by 5-HT through direct neurotransmission, paracrine transmission, and hormonal release of 5-HT, although the conditions under which each occurs are still unknown.

Relatively few examples of multiple targets for a modulator on a single cell have been described to the level of their distinct physiological pathways. Reported examples show that different targets may act in concert toward a common modulatory result or in qualitatively opposite directions and that they may be physically separated on the cell or grouped in a common area. In serotonergic raphe nuclei cells, 5-HT$_{1A}$ receptors localized on the soma and dendrites inhibit excitability, whereas 5-HT$_{1B}$ receptors located on axon terminals reduce transmitter release (Kia et al. 1996; Riad et al. 2000; Rumajogee et al. 2006; Sari et al. 1999). Serotonergic modulation of vagus neuron excitability in the rat dorsal motor nucleus (Browning and Travigli 1999; Fukushima et al. 2006), of sound-evoked responses in neurons in the inferior colliculus (Hurley 2006), and of gonadotropin-releasing hormone release from cultured neurons (Wada et al. 2006), are transduced through multiple receptors each with distinct physiological roles. Induction of some forms of 5-HT-elicited facilitation at Aplysia sensory to motor neuron synapses may result from differential delivery of 5-HT to the soma or synaptic processes of the sensory neurons (Casadio et al. 1999; Clark and Kandel 1993; Martin et al. 1997; Mauelshagen et al. 1998; Sherff and Carew 1999 and 2004). In the LG, spatial segregation of serotonergic responses and different sensitivities to applied 5-HT have been demonstrated, suggesting the involvement of more than one receptor type. Together these systems reveal an additional level of neuromodulatory control through the patterns of ligand delivery and differential activation of receptors and suggest that modulatory events should not be regarded as a simple binary phenomenon.

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