A Single Serotonergic Modulatory Cell Can Mediate Reinforcement in the Withdrawal Network of the Terrestrial Snail

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A cluster of 40 serotonergic cells in the rostral part of pedal ganglia of the terrestrial snail Helix lucorum was shown previously to participate in the modulation of withdrawal behavior and to be necessary during the acquisition of aversive withdrawal conditioning in intact snails. Local extracellular stimulation of the serotonergic cells paired with a test stimulus elicited a pairing-specific increase (the difference between paired and explicitly unpaired sessions was significant, \( p < .01 \)) of synaptic responses to test stimulation in the premotor interneurons involved in withdrawal. This result suggested participation of serotonergic cells in mediating the reinforcement in the withdrawal network. Intracellular stimulation of only one identified Pd4 cell from the pedal group of serotonergic neurons paired with a test stimulus also significantly increased (the difference between paired and explicitly unpaired sessions was significant, \( p < .05 \)) synaptic responses to paired nerve stimulation in same premotor interneurons involved in withdrawal. Morphological investigation of a cluster of pedal serotonergic neurons showed that only the Pd4 cell had branches in the parietal ganglia neuropile where the synapses of premotor withdrawal interneurons and of presynaptic neurons are located. The data suggest that a single serotonergic cell can mediate the reinforcement in the withdrawal network of the terrestrial snail. Patterns of responses of the Pd4 cells to tactile and chemical stimuli conform to the suggestion.

Key Words: reinforcement; neuromodulation; command neurons; serotonin; Helix.
INTRODUCTION

Changes in behavior that correspond to the activity of a single invertebrate nerve cell were described in the literature quite early (Wiersma, 1938; Willows, 1967; Nolen & Hoy, 1984). Well-known examples are lateral giant neurons in crayfish (Wiersma & Ikeda, 1964), Mauthner cells in fish (Eaton, 1984), and neurons controlling withdrawal in mollusks (Balaban, 1979). These cells were called command neurons and constituted a class of premotor interneurons whose intracellular activation elicits a goal-directed behavioral response similar to the responses evoked by adequate sensory stimuli (Wiersma & Ikeda, 1964). Nine giant premotor neurons (Balaban, 1979, 1983) located in the parietal and pleural ganglia of the snail *Helix* satisfy three criteria for command neurons introduced by Kupfermann and Weiss (1978). First, they respond to the presentation of a noxious tactile stimulus by a discharge which precedes the behavioral response (the “participation” criterion). Second, intracellular activation of one neuron releases a part of the withdrawal response (the “sufficiency” criterion). The last, the “necessity” criterion, is fulfilled for a component of the withdrawal behavior elicited by intracellular stimulation—this component disappears from the withdrawal response when the putative command cell is hyperpolarized. Thus, withdrawal responses in the snail are mediated by nine putative command neurons of pleural and parietal ganglia triggering head withdrawal, body withdrawal, pneumostome closure, and receiving common polymodal synaptic input (Balaban, 1979, 1983; Balaban & Zakharov, 1992).

In addition to putative command neurons for withdrawal behavior in *Helix* a group of serotonin-containing cells modulating the network underlying snail withdrawal behavior (Zakharov et al., 1995) was described. Firing in these neurons did not elicit any forms of behavior, but changed the behavioral responses evoked by noxious stimuli: such properties conform to a definition of modulatory cells. Extracellular stimulation of these serotonergic cells led to a short-term facilitation of synaptic and action potential responses in the putative withdrawal command neurons induced by noxious stimuli. Individual serotonergic cells responded with a stronger discharge to ipsilateral than contralateral stimulation and exhibited differences in receptive fields (Zakharov et al., 1995). Immunochemical investigation showed the presence of serotonergic terminals in the neuropile and somata layer surrounding putative command neurons of the parietal ganglia for withdrawal (Vehovszky et al., 1993), suggesting a direct interaction between serotonergic neurons and these command neurons.

It was shown previously in *Helix* that after treatment with the neurotoxin 5,7-dihydroxytriptamine (5,7-DiHT) selectively impairing the serotonergic neurons, context conditioning and associative food-aversion conditioning were impaired (Balaban et al., 1987; Zakharov & Balaban, 1991; Balaban & Bravarenko, 1993). This suggests that the serotonergic modulatory neurons can be involved in the reinforcement process underlying the development of conditioning.

In the present paper, we investigated the possible participation of the whole group and of individual serotonergic modulatory neurons in mediating reinforcement. In a model situation the synaptic inputs to premotor interneurons for withdrawal were paired or explicitly unpaired with the activation of modulatory cells. It appeared that extracellular activation of a group of serotonergic cells can serve as a reinforcement. Activity in a
single serotonergic cell can also mediate reinforcement in the withdrawal network of the terrestrial snail. Patterns of responses of Pd4 cells to tactile and chemical stimuli conform to this suggestion.

METHODS

Animals and Preparations

Experiments were carried out in adult Helix lucorum L. snails. All animals were kept in terraria. The isolated central nervous system (CNS) was used. Details are given elsewhere (Balaban et al., 1987; Maximova & Balaban, 1984).

Electrophysiology

Conventional microelectrode techniques were used for simultaneous intracellular recording from up to four identified neurons. The extracellular stimulation of cellular somata of modulatory neurons was accomplished by using a glass saline-filled extracellular stimulating electrode (inner diameter 200 μm) gently pressed to the desheathed ganglia surface by negative pressure. Parameters of stimulation were a frequency of 5 Hz and a pulse duration of 3 ms for 5 s. The effective voltage was variable (0.5–3 V) and was chosen individually in each experiment. The criterion for the threshold level of stimulation was a small (2±4 mV) depolarization in the withdrawal parietal interneurons, but the intensity never exceeded 3 V. The sizes of neurons in the stimulated parts of the pedal ganglia ranged from 30 to 150 μm (identifiable Pd1-4 neurons are much bigger). Using suction electrodes with fast green inside it was possible to estimate in four pilot experiments that under the opening of the suction electrode there are about 8–12 neurons. In six pilot experiments, the effectiveness of extracellular stimulation was investigated by recording intracellularly from neurons under and near the suction electrode. With the electrodes and voltage we used, spikes to each depolarizing pulse were evoked only in cells whose soma at least partly was under the electrode tip. At a distance of 100 μm from the electrode tip spikes were recorded only in 50% of recorded cells. A rough estimate is that we stimulated about 30–35 neurons on the ganglion surface.

Adult H. lucorum L. snails were dissected as previously described (Balaban, 1979). Physiological saline contained (mM): 80 NaCl, 4 KCl, 7 CaCl2, 5 MgCl2, and 10 Hepes (pH 7.6). The recording chamber for the isolated nervous system (volume 3 ml) was perfused at 0.3 ml/min. Identified withdrawal (command) premotor interneurones of the parietal ganglia (LPa3 and RPa3 but also LPa2 and RPa2) or pleural ganglia (LPI1 or RPI1) involved in triggering the withdrawal responses were penetrated with glass microelectrodes (3 M KCl, 10–20 MOhm). Test electrical stimuli were applied via polyethylene suction electrode to the intestinal nerve, which does not contain processes of the recorded cells. Stimulus intensity (0.5–3 V) and duration (3 ms) were adjusted to evoke the complex EPSPs of 4–10 mV in amplitude.

The experimental protocol for pairing experiments included testing, paired or explicitly nonpaired procedures, and posttesting. Long (5 or 20 min) intervals between test stimuli were used to diminish the low-frequency depression typical for the studied connections (Bravarenko et al., 1995). The reinforcing session consisted of five extracellular reinforcements of the intracellular tetanizations. Each extracellular tetanization
consisted of a 5-s train of regular 3-ms pulses with 5 Hz frequency, with the intensity adjusted 40 min before the beginning of the training session. Each intracellular tetanization consisted of a 10-s burst of 30-ms depolarizing pulses. The pulse frequency in each burst was 16 Hz and the pulse duration 30 ms. The intracellular current strength (5–15 nA) was suprathreshold. Spikes were on average evoked only by 35–40% of pulses at these frequencies.

Some of the experiments were performed in semi-intact preparations with preserved innervation of the skin (details in Balaban et al., 1987). The constant saline flow was directed from beneath the CNS to the periphery. Tactile stimulation was applied with the aid of an electromechanical device controlled by a stimulator. Punctate mechanical stimuli were applied with calibrated von Frey’s hairs, permitting delivery of pressures ranging from 6 (weak) to 68 (strong) g/mm². The intensity of tactile stimuli (von Frey’s hairs were used with a tip diameter of 0.1 mm) was estimated by measuring the force of each hair pressure on balances. A tactile stimulus of 68 g/mm² elicited withdrawal of tentacles and foot in intact snails and was used in electrophysiogical experiments as a “strong” tactile stimulation. Chemical stimulation (carrot juice, quinine) was applied to the lip.

Recordings were digitized (DIGI DATA 1200, Axon Instruments, U.S.A.) at 1 kHz and fed into an IBM PC compatible computer. Peak EPSP amplitudes were measured using “Axotape” software.

For statistical evaluations, the nonparametric Mann–Whitney rank sum test was used. “Significantly different” corresponds to at least \( p < .05 \), if not stated otherwise. Data were normalized to the first EPSP in the experiment. Data are presented as the mean and standard error of the mean.

A portion of the experiments were performed in snails with vitally stained serotonergic cells. In order to obtain selective vital staining of serotonergic cells, snails were injected 2 months before the experiment with selective for serotonergic cells neurotoxin 5,7-DiHT twice, with a 1-day interval, at 10 mg/kg of weight (details in Balaban et al., 1985). It was shown that 2 months after the injection the synaptic connections and normal levels of 5-HT were restored in the serotonergic cells (Gadotti et al., 1986; Vehovzsky et al., 1989), whereas the somata of the cells obtain for a lifetime a permanent red–brown staining (Balaban et al., 1985; Vehovzsky et al., 1989). Comparison of pigment labeling by 5,6-DiHT or 5,7-DiHT and immunolabeling techniques established that all the pigment-labeled neurons show serotonin (5-HT) immunoreactivity in Helix (Hernadi et al., 1989).

### Optical Recording

We performed optical recording with the fast-response probe RH-155 (NK-3041, Nikon) of the pyrazo-oxonol voltage-sensitive dyes group. The scheme of staining was selected as follows: 3 × 4 min of staining with saline containing RH-155 at a final bath concentration of 0.3 mg/ml (1 mg/ml stock solution in saline), at 4-min intervals (the dye was washed out) between staining periods. The recording was performed using transmitted light and 124 photodiodes with individual amplifiers (details in Tsau et al., 1993). The wavelength of the filter used was 710 ± 20 nm. The light passed through the preparation and then was projected via the optical system of the microscope on a photodiode array (124 detectors, Physical-Technical Institute, S. Petersburg). The microscope was equipped with a contact fluorescent objective (25×, 0.4 NA, LOMO). The amplifier outputs were digitized.
with a 12-bit analog-to-digital converter (Microstar Laboratories DAPL 3000A, 417 K samples/s) under the control of a Pentium-based computer. We used a frame rate of 1 kHz. The measured optical signal reflected the change in light transmission relative to its mean value ($T/T$). The software for optical data analysis was developed in the laboratory of Dr. L. B. Cohen (Yale University).

**Morphology**

Individual cells were stained by intracellular iontophoretic injection of a 6% solution of cobalt or hexamminecobalt chloride. Depolarizing pulses of 10 A (500 ms, 1 Hz) were delivered for 20–30 min. Several hours later, cobalt was precipitated as the sulfide with a saturated solution of hydrogen sulfide in saline. After 1–3 days of fixation with Carnoy’s fixative, the whole mount preparations were intensified with silver using procedures described by Davis (1982). The stained cells were drawn from whole mounts of ganglia cleared with methylsalicilite, with the aid of a drawing tube attached to a compound microscope. Sixteen giant parietal cells were successfully filled and 32 serotonergic pedal cells, 12 of them in nervous systems vitally stained (for better identification) with 5,7-DiHT 2 months before filling. Reconstruction of the morphology of the pedal group of serotonergic neurons was also made in animals with cells vitally stained with 5,7-DiHT (see above). Serotonergic cells stained by this method were intracellularly double-labeled with cobalt.

Retrograde filling with cobalt was carried out using a polyethylene suction electrode into which the stump of the nerve was sucked, and then cobalt was added to the pipette. In some experiments we used electrodes filled with 2 M KCl + 1% biocytin for recording from pedal serotonergic cells. After the experiment the preparation was processed using conventional techniques for biocytin (Scalia et al., 1997).

A total of 78 parietal and pedal neurons were traced using intracellular dye injection.

**RESULTS**

*Contingent Extracellular Activation of the Pedal Serotonergic Cells can Serve as a Reinforcement*

Previously published results showed the necessity of serotonergic cells for long-term behavioral sensitization and the elaboration of context conditioning and food aversion (Balaban et al., 1987; Zakharov & Balaban, 1991; Balaban & Bravarenko, 1993; Zakharov et al., 1995). The presence of serotonergic fibers surrounding the soma of withdrawal premotor interneurons with a dense network without synaptic specializations, suggesting a modulatory influence, was clearly demonstrated immunochemically (Vehovzsky et al., 1993). Therefore, we tested the assumption that serotonergic cells can mediate reinforcement. We performed experiments in which the EPSPs induced by nerve stimulation in the withdrawal interneurons were paired with local extracellular stimulation of serotonergic neurons located in the rostral part of the ipsilateral pedal ganglion. It should be noted that in 12 pilot experiments no significant short- or long-term effects on the amplitude of complex EPSPs in withdrawal interneurons were found when we extracellularly stimulated serotonergic cells located on the border of the visceral and right parietal ganglia.
In our experiments the test stimuli to the intestinal nerve were applied with a 20-min intertrial interval. A 20-min interval between test stimuli was selected in order to diminish habituation of complex EPSPs in premotor interneurons. During 5 h of experimentation the EPSP amplitude usually decreased to 85–90% of the level of the initial amplitude with the intertrial interval of 20 min (Maximova, unpublished). The first three test stimuli (pretesting) were followed by paired or explicitly unpaired procedures, and then five posttest stimuli were applied. The beginning of extracellular stimulation (5-s train duration, regular 3-ms pulses with 5-Hz frequency) of serotonergic cells during the paired procedure coincided with the beginning of the test stimuli, while during the explicitly unpaired procedure (in other preparations) a similar extracellular stimulation was given in the middle of the intertrial interval (10 min apart from test stimuli). The averaged data from 38 experiments (Fig. 1) showed a significant difference ($p < .01$ 100 min after the last reinforcing stimulus, Mann–Whitney rank sum test) between the amplitudes of EPSPs to test stimuli in premotor withdrawal neurons of paired and unpaired groups 60–100 min after the beginning of the reinforcing extracellular stimulation. These results suggested that pedal serotonergic neurons were capable of contingently increasing the amplitude of the withdrawal neuron responses to nerve stimulation. At a behavioral level this increase would result in facilitation of withdrawal responses similar to the one observed in our earlier experiments during context conditioning (Balaban & Bravarenko, 1993) and associative learning (Balaban et al., 1987).

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**FIG. 1.** (A) Averaged changes (means ± SEM) in amplitude of the complex EPSPs evoked in parietal withdrawal interneurons by stimulation of intestinal nerve paired (filled circles) and explicitly unpaired (open boxes, in different experiments) with extracellular activation (marked by arrows) of serotonergic pedal cells. The initial response was taken in all experiments as 100%.* $p < .05$, ** $p < .01$, Mann–Whitney rank sum test. (B and C). Examples of EPSPs before (solid line) and 90 min after the beginning of the training session (dotted line) are shown from the experiment with unpaired protocol (B), and from the experiment with a paired protocol (C).
One Modulatory Cell Can Mediate the Reinforcement

The experiments using extracellular stimulation described in the previous section cannot identify individual neurons involved in neuromodulation or provide important information about cellular mechanisms. Therefore, we used intracellular stimulation of individual cells in the rostral region of the pedal ganglia. The training procedure was changed in order to shorten the training session. Test stimuli were delivered with 5-min intervals before and after the pairing session. An increase in test stimulation frequency normally increases the habituation rate (Balaban & Zakharov, 1992). At a frequency of 1/5 min the response in parietal giant cells to test stimulation via the intestinal nerve usually habituates to 65–75% of the initial value (Bravarenko et al., 1995; Malyshev et al., 1997). A pairing session (Fig. 3A) consisted of five test stimuli with 2-min intervals and five “reinforcing” intracellular trains to the pedal neurons (Fig. 3B), which were given simultaneously with the test stimuli (paired procedure) or between test stimuli in a pairing session (explicitly unpaired group, Fig. 3A). The EPSP amplitude was not analyzed during the testing session (the gap in Figs. 2A and 3C) because the artifacts of tetanization in the paired procedure masked the form of the EPSPs (Fig. 2B). The “reinforcing” intracellular tetanization consisted of one 10-s duration train of 25- to 33-ms depolarizing pulses at 15–20 Hz. The current strength (5–10 nA) was suprathreshold. Giant parietal withdrawal interneurones (mainly LPa3 and RPa3) and one of the pedal serotonergic cells were penetrated with one or two glass microelectrodes. The second electrode in the pedal cell was used for intracellular tetanization and injection of biocytin (Fig. 3B). In most experiments, the

![Graph A](image1.png)

**FIG. 2.** (A) Changes in amplitude of complex EPSPs evoked in parietal withdrawal interneurons by stimulation of intestinal nerve paired and explicitly unpaired with intracellular activation of serotonergic pedal cells other than Pd4 (10 cells in 10 different experiments). The initial response was taken in all experiments as 100%. The training session is marked by breaks in axes and an arrow. (B) Example of pairing of the test input evoking EPSPs in parietal withdrawal interneurons with extracellular tetanization.
FIG. 3. Changes in the amplitude of complex EPSPs evoked in parietal withdrawal interneurons by stimulation of intestinal nerve paired and explicitly unpaired with intracellular activation of the Pd4 cell. (A) diagram of the experiment; (B) scheme of recording and experimental set-up; (C) averaged results (means ± SEM); *p < .05, **p < .01, Mann-Whitney rank sum test. The initial response was taken in all experiments as 100%. The training session is marked by breaks in axes and an arrow. In the inset examples of EPSPs before (solid line) and 30 min after (dotted line) training session from the same experiment are shown.

training procedure was repeated twice: one paired and one unpaired session. In total 27 animals were used for experiments. In 12 of them snails with serotonergic cells previously vitally labeled by 5,7-dihydroxytriptamine (Balaban et al., 1985) were used. Brown pigmentation characteristic of 5,7-DiHT-labeled cells allowed us to be sure that a serotonergic cell was impaled in these preparations. In most experiments, pedal cells were filled with biocytin after the experiment to verify the morphology of the recorded cell.

To our surprise, we never observed any modulatory or pairing-specific effects in the experiments, in which we tetanized an unidentified small serotonergic pedal neurons or serotonergic Pd2 cells (Fig. 2A). The difference between responses in paired and unpaired situations was close to 0 in 17 snails and never exceeded the standard error of the mean on averaging. Only when intracellular tetanization of Pd4 cells was used as a reinforcement did we observe an increase in EPSP amplitude during the paired procedure (n = 10 snails; Fig. 3C) relative the experiments with unpaired stimulation of Pd4 cells (n = 6, same snails; Fig. 3C). A significant difference was observed immediately after the last tetanization (p < .05, Mann-Whitney rank sum test; corresponding values were compared in experiments with paired and unpaired procedures). Thirty minutes after the pairing session the
difference was even more significant ($p < .01$), and up to the 50th min the difference between paired and explicitly unpaired situations was significant (Fig. 3C). In general, the results were similar to those obtained in experiments with extracellular stimulation of the serotonergic neurons (Fig. 1). Thus, intracellular stimulation of only one Pd4 cell can mediate a pairing-specific increase in the amplitudes of the EPSPs in the parietal giant neurons controlling withdrawal behavior.

**Morphology of Serotonergic Cells**

Intracellular staining of the rostral pedal cells with Co$^{2+}$ (46 neurons) or biocytin (32 cells) revealed processes leaving the ganglia via cutaneous nerves. Stained neurites of only one cell (Pd4) were observed in the neuropile of a parietal ganglion where an extensive plexus of neurites of premotor withdrawal interneurons is located (Fig. 4C), as well as presynaptic cells sending processes in the intestinal nerve (Arakelov et al., 1991).

Using the method of retrograde transport of Co$^{2+}$, we traced neurons sending axons from the pedal ganglia to the neuropile of the parietal ganglia. Staining via parietopleural

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**FIG. 4.** Morphology of parietal and pedal cells. (A) Location of cells revealed with cobalt tracing via the pleuroparietal connection (PIParC). Filled circles, cells proved to be serotonergic in preparations pigment-labeled with 5, 7-DiHT; open circles, nonserotonergic cells. (B) Distribution of branches of parietal withdrawal interneuron No. 3 (triggering pneumostome closure and body withdrawal) revealed by intracellular injection of cobalt. (C) Distribution of branches of the Pd4 cell revealed by intracellular injection of cobalt. Note overlapping of processes of parietal giant and Pd4 in the neuropile of parietal ganglion.
connections consistently revealed the Pd4 serotonergic cell, one to two randomly observed small serotonergic cells in the rostral part of pedal ganglia, a couple of identifiable large nonserotonergic cells, and two groups of small nonserotonergic cells (Fig. 4A). Taking into account that processes of only Pd4 cells overlap with processes and the putative synaptic region of the giant parietal (command) cells in neuropile of the parietal ganglia (Figs. 4B and 4C), it is logical to assume that mainly the Pd4 cell exerts the described contingent changes in amplitude of synaptic input of withdrawal interneurons. It should be noted that the Pd4 cell can be individually identified in snail embryos at the final stage of development, at the stage when the first withdrawal responses to noxious stimuli can be observed in the embryos (Ierusalimsky, unpublished).

Participation of the Pd4 Cell in the Control of Withdrawal Behavior

It is known from earlier studies that the cells located in mediorostral part of the pedal ganglia, on both dorsal and ventral sides, contain serotonin (Sakharov, 1974; Balaban et al., 1985; Zakharov et al., 1995). It was shown that distribution of 5-HT-containing cells in the central ganglia of adult Helix using an immunochemical method and vital pigment labeling by 5,7-DiHT (see Methods) coincides. The most important fact for the present investigation is that all pigment-labeled cells show the 5-HT immunoreactivity (Hernadi et al., 1989). Schematically, the location of serotonergic cells on the dorsal surface of pedal ganglia obtained by those two methods (our results using the 5,7-DiHT and the literature) are presented in Fig. 5A. In the rostral part of the pedal ganglia were found serotonergic cells only. Each pedal ganglion contained 40–50 serotonergic cells in the rostral region and several small clusters on the ventral side. This area was the only one effective for eliciting facilitation of the withdrawal responses using small intensity extracellular stimulation (Zakharov et al., 1995). These results suggested that the pairing-specific effects of extracellular stimulation of pedal cells on the synaptic input of the withdrawal command neurons could be attributed to serotonergic cells.

Using voltage-sensitive dyes and an array of photodiodes, we tried to characterize the spontaneous and evoked activity of the rostral serotonergic group in pedal ganglia as a whole (n = 12 experiments with a total of 480 recorded neurons). In all these experiments the serotonergic cells were pigment-labeled with 5,7-DiHT, and it was possible to record a majority of cells belonging to the serotonergic group by focusing on them and saving the image into a video file. The majority (76 ± 5%) of the recorded pigment-labeled cells in the rostral part of the pedal ganglia were spontaneously active. In the experiment presented in Figs. 5B and 5C, the activity of 41 serotonergic cells pigment-labeled with 5,7-DiHT was recorded from the region containing 50–60 cells accessible for optical recording. Each trace on Fig. 5C represents the activity of an individual neuron. The location of all neurons was established by software for optical recording which estimated the size of each neuron and the video imaging technique. It should be noted that using the absorption technique we cannot distinguish a dorsal or ventral location for small neurons. Thirty-eight neurons recorded in this experiment responded to stimulation of the second cutaneous nerve with excitatory responses (Fig. 5C). Cell 23 responded with a transient inhibition of spontaneous activity. Cells 40 and 112 (numbers correspond to a central photodetector) showed no changes in activity exceeding 20% of the initial frequency
FIG. 5. Optical recording of the activity of serotonergic pedal cells with the membrane potential-sensitive dye RH155. (A) Map of the location of serotonergic cells on the dorsal surface of pedal ganglia. Square, a region of optical recording is marked. (B) Reconstruction of the size and location of cells recorded optically (activity shown on C) on the photodiode array. (C) Raster diagram of spontaneous activity and response to nerve stimulation (arrow) in serotonergic cells. PC, pedal commissure; St, statocyst; L, RPdG, left, right pedal ganglion.
FIG. 5—Continued

↑ 2nd n. cutaneus, descending branch,
taken as a criterion. Similar results were obtained in another 11 experiments. In 4 of 12 experiments bursting spontaneous activity of the recorded serotonergic cells was evident.

Optical recording of the activity of at least 70% of the cluster of serotonergic rostral pedal cells confirmed the assumption derived from the electrophysiological experiments that these cells respond with a spike discharge to stimulation of the second cutaneous nerve conveying the noxious information from the skin (Balaban & Zakharov, 1992).

It has been previously shown that some pedal serotonergic cells respond to a short (0.1 s duration) tactile stimulus in semi-intact preparations with an increase of spontaneous firing for at least 10 s (Zakharov et al., 1995). Optical recording in the isolated CNS allowed us to estimate that 54 ± 6% (12 experiments, 480 cells) of the recorded cells increased their firing frequency in response to cutaneous nerve stimulation for at least 10 s, with a criterion of a 20% increase of the initial frequency (21 of 41 cells in Fig. 5C). The Pd4 neuron behaved as a typical representative of this group (Fig. 5C, detector 68).

In response to the first tactile stimulation in a series of regularly applied tactile stimuli the PD4 cell (recorded intracellularly in a semi-intact preparation) showed a significant increase in background action potential frequency (Fig. 6A, interstimulus interval, 20 s). The maximum frequency increase in Pd4 cells correlated with the maximum action potential response in the command withdrawal neurons (Fig. 6A). Interestingly, the decrease in firing frequency in the Pd4 cell corresponded to the decrease in a number of spikes in the command neuron (Fig. 6A). Similar results were obtained in six semi-intact preparations.

Intracellular activation of the PD4 cell with a strong current in the isolated CNS (similar results in seven preparations) resulted in a prolonged depolarization, the appearance of action potentials, and a potential characteristic for electrical coupling (Fig. 6B). It should be noted that electrically coupled neurons were described in the pedal serotonergic cluster.

![FIG. 6. Properties of the Pd4 cell. Semi-intact preparation. (A) Simultaneously recorded responses of the withdrawal LPa2 interneuron and the ipsilateral serotonergic LPd4 cell to a regular tactile stimulation (intensity 0.6 g, tip diameter 0.1 mm) of the skin on the semi-intact preparation (arrows). Calibration, 10 mV, 10 s. (B) Recruitment of activity in a pedal serotonergic neuron evoked by its strong intracellular stimulation marked by a bar. Note positive deflections which are typical for spikes in electrically connected cells. Calibration, 10 mV, 3 s.](image-url)
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(Zakharov et al., 1995). This result suggests the existence of positive feedback in the network in which the Pd4 cell is included.

Intracellular stimulation of the PD4 cell in semi-intact preparations during regular presentation (1/20 s) of a weak tactile stimulus to the skin elicited facilitation of responses of withdrawal interneurons in seven of nine preparations. For example, in Fig. 7A action potentials were generated in withdrawal interneurons in response to regular tactile stimuli during intracellular activation of the PD4 cell instead of subthreshold EPSPs, which were recorded before the intracellular stimulation.

It was interesting to determine whether suppression of spontaneous activity in the PD4 cell would influence the withdrawal network activity. Unfortunately, parietal command cells have a high spiking threshold and are usually silent. We failed to observe any changes in transmembrane potential or responses to weak nerve stimuli (which do not elicit sensitization) in these cells after hyperpolarization of the Pd4 cell (four experiments, results not shown). We have not tried to use stronger nerve stimulation as a test input because it activates the whole group of serotonergic neurons, and interpretation of any changes observed under hyperpolarization of Pd4 cells will be arbitrary: changes may

FIG. 7. Effects of intracellular stimulation of the Pd4 cell on a semi-intact preparation. (A) Intracellular depolarization of the Pd4 cell provokes spike responses to regular tactile stimuli in withdrawal interneuron LPa2 in spite of the usual habituation of the responses. (B) Effect of hyperpolarization of the left pedal cell No. 4 (LPd4) on the spontaneous firing of the left pleural giant cell involved in head withdrawal. The pedal cell was gradually hyperpolarized from the resting potential (RP) to a level when no firing occurred. Note correspondence between the action potential frequency in the Pd4 cell and in the giant pleural cell. Calibration, 10 s, 10 mV.
be due to lack of spikes in the Pd4 cell or to depression of activity in electrically connected neurons.

In five experiments we tried to find effects of a long-term (minutes) hyperpolarization of the ipsilateral Pd4 cell on the spontaneous firing of the pleural giant cell, which is a member of a group of withdrawal command neurons, and its function is to control the head withdrawal (Balaban, 1979). The pleural giant cells demonstrated spontaneous action potential activity at a slow rate in these experiments (Fig. 7B). Hyperpolarization of the Pd4 cell, which prevented its spiking, elicited a decrease in spontaneous firing of the giant pleural cells (Fig. 7B). Release of hyperpolarization triggered the appearance of spikes in the withdrawal interneurons, and the depolarization elicited the appearance of additional spikes in the Pd4 cell and in the pleural withdrawal interneuron (Fig. 7B). This result, repeated in all five experiments in which there was an initial spontaneous firing in pleural cells, suggested that the Pd4 cell is involved in maintaining a certain level of excitability in the group of withdrawal interneurons. We cannot exclude participation of other serotonergic cells sending processes to the pleural ganglion or electrically connected to the Pd4 cell.

The above observations of Pd4’s response to noxious tactile stimuli (Figs. 4C and 5A) are consistent with Pd4’s proposed role in facilitating withdrawal reflexes. To address whether other classes of noxious and nonnoxious stimuli can also activate Pd4, we tested the effect of chemical stimuli (nonnoxious—food—and noxious—liquid with quinine) on Pd4’s activity. The Pd4 cells do not respond with a significant (more than 20% of initial frequency) increase in firing to application of a food stimulus to the lip in semi-intact preparations (six animals; the average frequency after the stimulus application was 112 ± 8% of initial frequency, Fig. 8B). Recorded simultaneously (as a control of a preparation condition) giant metacerebral cell 1 responded to the same stimuli with a significant increase in firing frequency. Application to the lip of quinine, known to elicit withdrawal responses in Helix, elicited a distinct discharge in all seven preparations tested (Fig. 8A). The average increase relative to the prestimulus frequency for PD4 cells was 480 ± 54% (n = 7; frequency was measured 40 s before and 40 s after the beginning of stimulus application). Thus, the Pd4 cell responded with a discharge to noxious chemical and tactile stimuli and did not respond to nonnoxious food stimulation, which conforms to a suggestion about its participation in modulation of withdrawal behavior.

**DISCUSSION**

*Role of Serotonin in Withdrawal Behavior*

Numerous studies in several gastropod species have indicated that the neurotransmitter 5-HT has a modulatory role in feeding behavior (Kupfermann & Weiss, 1981; Gelperin, 1981) as well as withdrawal behavior. Serotonin also plays an essential role in modulation of withdrawal reactions in mollusks (Kandel & Schwartz 1982; Balaban et al. 1987; Glanzman et al., 1989).

Serotonin-containing cells are described in the pedal ganglia of practically all gastropod species (see Croll, 1988, for discussion). Unlike the giant cerebral serotonergic cells whose involvement in the control of feeding behavior has been described in many species (Weiss & Kupfermann, 1976; Granzow & Kater, 1977; Gillette & Davis, 1977; Gelperin,
FIG. 8. Responses of the giant serotonergic metacerebral cell (LMtC) and the right pedal cell 4 (RPd4) to the application to the lip of a drop of quinine (A, $10^{-4}$ M) and of a drop of a carrot juice (B). The stippled bar marks the timing of stimulus application; the same semi-intact preparation, 40-min interval and washout between applications. Calibration, 10 mV, 5 s.

1981, etc.), including Helix (Weiss & Kupfermann, 1976; Galanina et al., 1986), the behavioral role of pedal serotonergic neurons has not been extensively investigated.

There are three groups of serotonergic neurons described in Helix (Sedden et al., 1968; Zakharov & Balaban, 1987, 1991). The cerebral group of serotonergic cells modulates feeding behavior (Balaban, 1991). A group of serotonergic cells located on the border of the left parietal and visceral ganglion may be involved in the control of heart and intestinal tract activities (Balaban, unpublished), although their precise function is still unknown. Pedal serotonergic cells are involved in the modulation of withdrawal behavior (Zakharov et al., 1995) and the possible control of the locomotion (suggested by their branching pattern).

The role of serotonin in the withdrawal behavior of terrestrial snails was investigated by manipulating 5-HT levels either (1) directly, by increasing the concentration of 5-HT in the medium bathing the CNS, or (2) indirectly, by selectively destroying serotonergic nerve terminals using 5,7-DiHT injections into intact animals (Balaban et al., 1987; Vehovszky et al., 1989). An increase in 5-HT concentration up to $5 \times 10^{-3}$ M in semi-intact preparations elicited sensitization of behavioral responses to noxious stimuli, with a corresponding increase in synaptic responses in the withdrawal interneurons (Balaban et al., 1986; Zakharov & Balaban, 1991) and changes in the duration of action potentials in sensory neurons responding to noxious stimuli (Balaban, 1987). These data suggested that serotonin is a facilitating modulatory transmitter for withdrawal behavior.

Injection of the selective neurotoxin 5,7-DiHT resulted in changes in the dynamics of the withdrawal reactions in intact snails and corresponding changes in the number of
spikes in the withdrawal command neurons. An increase in the amplitude of pneumostome closure and a corresponding increase in the action potential discharge in withdrawal interneurons during rhythmic tactile stimulation of the skin were always observed in control animals (Balaban, 1983, 1991). However, this sensitization was absent in 5,7-DiHT-injected snails at both the behavioral and cellular levels (Balaban et al., 1987; Balaban, 1993; Balaban & Bravarenko, 1993). These data confirm the suggested modulatory role for serotonin in the withdrawal behavior.

**Pedal Serotonergic Cells Constitute a Functional Neuromodulatory Group**

Each group of serotonergic cells has its target areas where most processes of the cells are branching. With the exception of the well-studied giant metacerebral serotonergic cell (Osborne, 1984), cerebral and parietovisceral serotonergic cells have not been investigated in detail. The morphology of the pedal serotonergic cells described in the present paper suggests that they represent several heterogeneous populations. Some cells send a single process to the peripheral nerves (V. Ierusalimsky, unpublished observations), while others send processes and branches to the neuropile of the pleural ganglia. Only the Pd4 cell from this group sends processes to the neuropile of the parietal ganglia (Fig. 4). Noxious tactile stimuli elicit an increase in the background spiking frequency in the serotonergic modulatory neurons and a corresponding increase in the stimulus-evoked action potential responses of the withdrawal interneurons. Although the entire group of serotonergic cells responds to noxious stimuli, most individual cells respond with a stronger discharge to ipsilateral than contralateral stimulation and exhibit differences in receptive fields. Sensory inputs to putative modulatory cells and their characteristic background firing are worth special attention. In order to facilitate withdrawal reactions, a putative modulatory neuron should receive information concerning each noxious stimulus which is delivered to the animal. In addition, changes elicited by such a stimulus must last for tens of seconds because behavioral facilitation usually lasts that long. It appears that responses of the investigated pedal serotonergic cells to noxious tactile stimulation are compliant with these conditions. These cells can be activated by stimulation of any part of the animal’s skin, but only by a strong noxious stimulus which also evokes a behavioral sensitization (Figs. 5 and 6). Only some cells respond to adequate stimulation of a given area, whereas intrinsic interconnections (Fig. 6B) within the group recruit other members in the case of a strong noxious stimulus. The weakness of electrical coupling between serotonergic cells (Zakharov et al., 1995) prevents recruitment of all cells in the group into responses to relatively weak stimuli, which are not dangerous for the snail. The tonic feature of their response to external stimuli is consistent with their role in the facilitation of the withdrawal response (Figs. 6A and 8). Extracellular stimulation of the investigated serotonergic cells led to facilitation of action potential responses to noxious stimuli in the putative command neurons for withdrawal behavior, suggesting their reinforcing role in this behavior.

Thus, pedal serotonergic cells apparently function as a modulatory system for withdrawal behavior, which facilitates synaptic responses in the underlying network.

**A Single Cell Can Be Responsible for Modulation of Behavior**

Contingent activation of only one pedal serotonergic cell (Fig. 3) produces facilitation of the synaptic inputs to the withdrawal interneurons, which is similar to the facilitation
induced by exogenous serotonin (Balaban et al., 1986, 1991; Zakharov et al., 1995). It is also similar to the facilitation elicited by the contingent extracellular activation of the group of pedal serotonergic neurons (Fig. 1). These results suggest that the activation of an individual pedal serotonergic modulatory Pd4 neuron can mediate reinforcement. We cannot exclude the possibility that this activation of a single cell activates other serotonergic cells as well.

No synaptic potentials were recorded in parietal cells during activation of serotonergic neurons. This may be due to the remoteness of synaptic connections or nonsynaptic release of a transmitter from varicosities which are characteristic of the serotonergic cells in Helix (Osborne, 1984). Immunochemical characterization of serotonergic fibers surrounding soma of premotor giant parietal cells and lack of synaptic specializations suggest a modulatory function of the serotonergic input (Vehovzky et al., 1993). We cannot completely exclude the existence of intermediate neurons. However, it seems unlikely, because the effect of direct contingent application of serotonin on synaptic transmission was shown in cultured identified neurons (Bao et al., 1998), and serotonin was shown to be necessary for long-term facilitation in molluscs (Glanzman et al., 1989). Overlapping of neurites of the Pd4 cell and giant parietal cells (Fig. 4) suggests that serotonin released from the Pd4 cell can affect sensory neurons presynaptic to the withdrawal interneurons or synapses between sensory neurons and withdrawal interneurons shown to be present in the same neuropile (Arakelov et al., 1991).

Participation of individual modulatory cells in modifications of behavior was shown in different invertebrates. Intracellular stimulation of identified cerebral Aplysia CB1 neurons produced facilitation of the EPSPs from siphon sensory neurons to motor neurons, suggesting participation of these individual serotonergic cells in mediation of presynaptic facilitation (Mackey et al., 1989). There are several published examples of identified neuromodulatory interneurons which serve a reinforcing function during associative learning. The octopaminergic VUMmx1 neuron, which mediates the reinforcing function of rewards in honeybees during olfactory conditioning, innervates most principal brain neuropiles with axodendritic arborizations. This neuron responds to sucrose (reward) with long-lasting excitation, and its depolarization substitutes for the reward in single-trial conditioning (Hammer & Menzel, 1995). It was clearly shown in cultured Aplysia neurons that temporal pairing of presynaptic activity and serotonin application enhances facilitation at sensory–motor neuron synapses (Eliot et al., 1994; Bao et al., 1998). Activation of an identified modulatory cell (slow oscillator) in Lymnaea stagnalis elicited associative enhancement of fictive feeding response (Kemenes et al., 1997).

The neuromodulatory serotonergic cell Pd4 in Helix innervates neuropiles of pedal, pleural, parietal, and visceral ganglia. It responds with long-lasting excitation to short noxious stimuli, which serve as a reinforcement in aversive conditioning (Balaban et al., 1987; Zakharov et al., 1995). Intracellular depolarization of this cell changed the effectiveness of synaptic input in withdrawal interneurons, while the hyperpolarization of the Pd4 cell decreased the rate of spontaneous activity in interneurons (Fig. 7). Conditional depolarization of the Pd4 cell elicits a pairing-specific increase in the amplitude of synaptic inputs to premotor withdrawal interneurons (Fig. 3), suggesting an increase in behavioral response. We suggest here that a single Pd4 cell can trigger the aversive reinforcement in the snail. This cell can be viewed as a “delegate” neuron, representing the activity of
a large group of modulatory serotonergic cells receiving sensory inputs from all parts of the body, but which do not send processes to the target (parietal) neuropile.

Observed differences in paired versus unpaired treatments may be attributed to an activity-dependent increase in presynaptic release due to the fact that serotonin application alone is effective in the isolated nervous system and in cultured neurons of molluscs (Eliot et al., 1994; Bao et al., 1998). Still, a prolonged depolarization in a postsynaptic neuron also may contribute to the potentiation of response due to coincidence of activity in pre- and postsynaptic neurons in a Hebbian way. Additional experiments are necessary for distinguishing these possibilities.

REFERENCES


