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Non-Synaptic Integration of the Cell Bodies of Neurons into the Central Nervous System of the Snail

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Single neurons (soma and proximal process) were isolated from the serotonergic (5-HT-ergic) PedA cluster in experiments on the pond snail *Lymnaea stagnalis*, and changes in the electrical activity of isolated neurons were observed during repeated movement of these cells towards and away from the surface of the CNS. The position of cell bodies of 5-HT-ergic neurons had excitatory effects on the isolated neuron. This effect was maximal (at 10^{-8}–10^{-7} M 5-HT) when neurons were brought close to the PedA cluster and were further enhanced by addition of the 5-HT precursor 5-hydroxytryptophan at concentrations of $(1–2)\cdot 10^{-4}$ M. The results obtained here provide evidence 5-HT-ergic neurons cooperate during 5-HT-dependent behaviour, this being based on excitatory interactions at the level of cell bodies.

KEY WORDS: Serotonin, 5-hydroxytryptophan, isolated neurons, serotonergic neurons, *Lymnaea stagnalis*.

This report describes results obtained from studies of the mechanisms underlying the development of the behavioral state known as serotonergic arousal. This state is induced in a number of mollusks (*Aplysia*, *Clione*) by the metabolic serotonin precursor 5-hydroxytryptamine (5-HTP). Feeding-associated elements of locomotion and behavior are enhanced during serotonergic arousal, while passive-defensive behavior is suppressed [8, 16, 18, 21]. At the cellular level, the most marked correlate of the behavioral effects of 5-HTP is a sharp increase in the activity of the serotonergic medial pedal ganglion neurons, which are involved in mediating locomotor programs [4, 15].

In the mollusk model used here (the pond snail), the medial neurons of the left and right pedal ganglia, which form paired PedA clusters [22], are also involved in controlling locomotion. PedA cells produce serotonin (5-HT) and, along with intracentral connections, send peripheral processes to locomotion effectors [23, 24]. The effect of 5-HTP in the pond snail is to change the ciliary mode of locomotion to the muscular [11, 19], with increases in the 5-HT level in the CNS [17] and the electrical activity of PedA neurons [5, 9]. Similar changes in the mode of locomotion and 5-HT levels were induced in the pond snail by loading (weighting of the shell) [17], which is evidence for the adequacy of this pharmacological model of serotonergic arousal.

We have previously found that excitation of PedA neurons can be prevented when the conversion of 5-HTP to 5-HT is suppressed by a tryptophan decarboxylase inhibitor [10]. It became clear that exogenous 5-HTP has no direct excitatory effect on PedA cells; the cause of the excitation was additional intracellular 5-HT synthesis. This was supported by experiments on isolated PedA neurons [1], which also showed that the development of excitation induced by 5-HTP involves the mechanism of vesicular secretion [2]. The results provide the basis for the hypothesis that in the presence of precursor, the neuron becomes excided because of additional somatic secretion of transmitter. This led to the suggestion that the immediate pericellular space may contain effective 5-HT concentrations which might act on depolarizing serotonin autoreceptors.

The verification reported here not only supported this suggestion, but also yielded evidence that neurotransmitter released by the cell body is significant in terms of the activity of neighboring neurons. The results presented here have been presented in part at an international conference [13].

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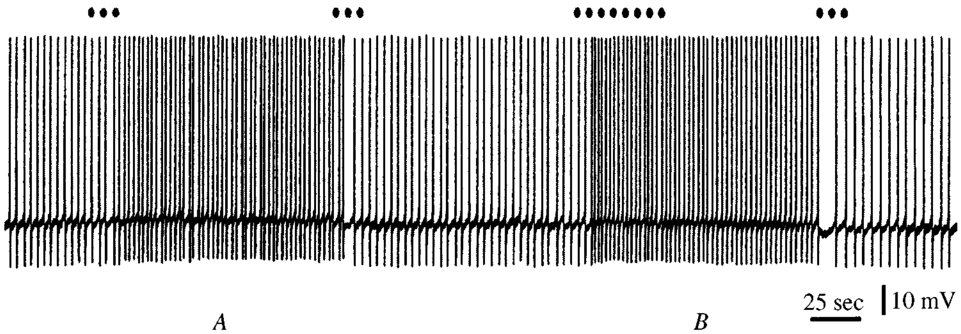

Fig. 1. Electrical activity of an isolated PedA neuron on approximation to the surface of the PedA cluster (*A*) and to the tip of a 5-HT-containing capillary (*B*). Dots show mechanical movements of the neuron.

METHODS

Studies were performed using adult greater pond snails of the species *Lymnaea stagnalis* (*Pulmonata*, *Basommatophora*), grown in aquarium cultures. Isolated CNS preparations lacking the buccal ganglia and with section of the left cerebropedal and cerebropleural ganglia connectives were treated with pronase E (Sigma) at a concentration of 4 mg/ml for 35 min and washed with Ringer's solution for snails. CNS preparations were then placed in the 1.5-ml working chamber and a flow of Ringer's solution (50 mM NaCl, 1.6 mM KCl, 4 mM $CaCl_2$, 8 mM $MgCl_2$, and 10 mM tris, pH 7.4) was established. The flow rate was 0.7–1.0 ml/min and was maintained throughout experiments except when stated.

Serotonin (5-hydroxyl-L-tryptamine, 5-HT) and its precursor (5-hydroxy-L-tryptophan, 5-HTP, both from Sigma) were added to the flow or were introduced into the chamber with a micropipette. The neuron membrane potential was measured with an intracellular microelectrode with R = 20–60 MΩ filled with KCl (0.08–3 M); signals were passed to a Micromed amplifier, then to an analog-to-digital converter, and were then recorded on an IBM PC 486DX4-S personal computer.

The general experimental protocol was as follows. After the flow was started, one PedA cell was isolated as described by D'yakonova [3]. This was performed by inserting the recording electrode into the cell body; this was slowly moved to one side until the neurite was completely separated from the neuropil. The isolated neuron was initially established in a position 0.5–1.0 mm from the pedal ganglia. The microelectrode was used both for further movement of the cell and for recording electrical activity. When movements were made, the proximal process of the neuron was generally stretched along the flow and was not subjected to strong mechanical deformations. The state of the cell was assessed during mechanical manipulations from its baseline activity. During experiments, isolated neurons were moved under visual control from a distant point to a selected area of the surface of the pedal ganglia to the point to contact, sometimes with gentle touching. In some experiments, the neuron was also brought to the tip of a capillary filled with 10^{-3} M 5-HT. The blunt end of the capillary was filled with mercury to prevent evaporation.

RESULTS

Changes in the Activity of Isolated Neurons Close to the CNS and 5-HT-Filled Capillaries. Approximation of neurons isolated from the PedA cluster to the pedal ganglia led to changes in activity. Testing on approximation was performed 0.5–2 h after starting the flow and was repeated several times (usually over 10–20 min, sometimes over 3–4 h). In the vast majority of cases (36 of 42 cells, 31 CNS preparations), the spike frequency of isolated neurons approximated to the surface of the ganglion in the zone corresponding to the PedA cluster increased to 112–1000% (mean 280%) of baseline (Fig. 1). However, there were exceptions, where there were no significant changes in frequency (three cases) or where the frequency decreased (three cases). When the neuron was repeatedly approximated to the same point, it behaved the same way as it did the first time.

Approximation of isolated neurons from the PedA cluster to one of the cerebral ganglia in the region of the body of a 5-HT-ergic metacerebral cell also led to increases in spike frequency (four cases of four). This result was obtained when neurons were approximated to other ganglia containing 5-HT-ergic cells – the visceral and right parietal ganglia – but not with the pleural ganglia (two cases of two).

Movement along the surface of the pedal ganglia produced regular changes in the activity of isolated neurons, such that each point on the surface corresponded to a characteristic spike frequency. The maximum frequency was invariably reached in the zone of the PedA cluster (Fig. 2, left part). Movement of isolated neurons in a flow of Ringer's solution washing the CNS preparation produced

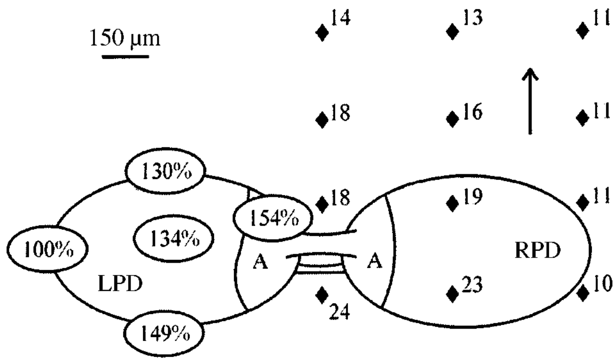

Fig. 2. Changes in the activity of an isolated PedA neuron depending on its position relative to the pedal ganglion. The LPD map shows the activity of the isolated neuron at different parts of the dorsal surface (mean of seven experiments, % of the frequency at the lateral margin of the ganglion). The RPD shows the results of an experiment in which the isolated neuron was moved along the flow. The number of spikes recorded in 2 min is shown for each point. The arrow shows the direction of the flow. A = PedA cluster. LPD = left pedal ganglion. RPD = right pedal ganglion.

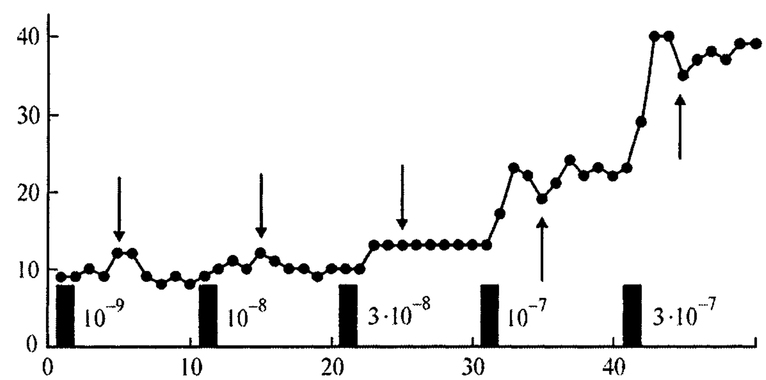

Fig. 3. Responses of an isolated PedA neuron to approximation to the PedA cluster in the presence of increased 5-HT concentrations. The abscissa shows time, min; the ordinate shows the number of spikes. Each line on the plot shows the number of spikes in 1 min. Each arrow shows the minute at the beginning of which movement of the neuron towards the surface was started. The time taken to move the neuron (removal) was 0.5 min; the time at the ganglion surface between approximation and removal was 1.5 min. Black bars along the abscissa show the start of addition of a new 5-HT concentration to the chamber (M).

changes in spike frequency which could be seen at large distances (up to 600 μm) from ganglia (Fig. 2, right part). When isolated PedA neurons were moved close to the tips of 5-HT-containing capillaries, spike frequencies also increased (eight of eight cases, Fig. 1). The same occurred in the single experiment in which a neuron responding to approximation to the PedA cluster with a decrease in spike frequency was brought close to the 5-HT-containing capillary.

Changes in Activity of Isolated Neurons Close to Pedal Ganglia in the Presence of Exogenous 5-HT. Addition of 5-HT ($2 \cdot 10^{-9}$ M) to the flow significantly altered the spike frequencies of isolated neurons. At 10^{-8}–10^{-7} M, 5-HT induced increases in neuron spike frequency similar to those which could be obtained when neurons were brought close to ganglia. Periodic approximation of isolated neurons to pedal ganglia in conditions of gradually increasing 5-HT concentrations in the flow (two cases) showed that after exogenous 5-HT had yielded some threshold, the spike frequency stopped increasing and could even decrease when the cell was approximated to pedal ganglia (Fig. 3). At $5 \cdot 10^{-5}$ M 5-HT, approximation of isolated neurons to the PedA cluster produced no increases in electrical activity. Conversely, these conditions produced reductions in spike frequency (in six of 10 cases).

The action of 5-HT in capillary tips was essentially equal to that of 10^{-8}–10^{-7} M 5-HT. This 5-HT concentration in the flow blocked the responses of isolated neurons to approximation to the 5-HT-containing capillary.

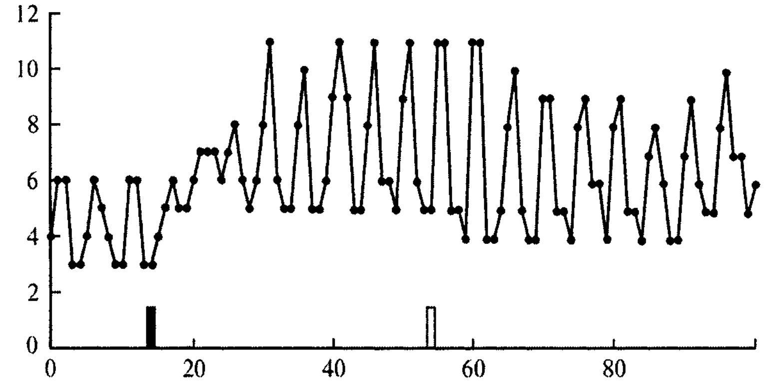

Fig. 4. Responses of an isolated PedA neuron to approximation to the PedA cluster in the presence of 5-HTP at a concentration of $2 \cdot 10^{-4}$ in the flow. The neuron was moved as in the experiment described in Fig. 3 every 6 min starting at time point 0 min. The black bar on the abscissa shows the start of addition of 5-HTP to the chamber; the white bar shows the start of washing. For further details see caption to Fig. 3.

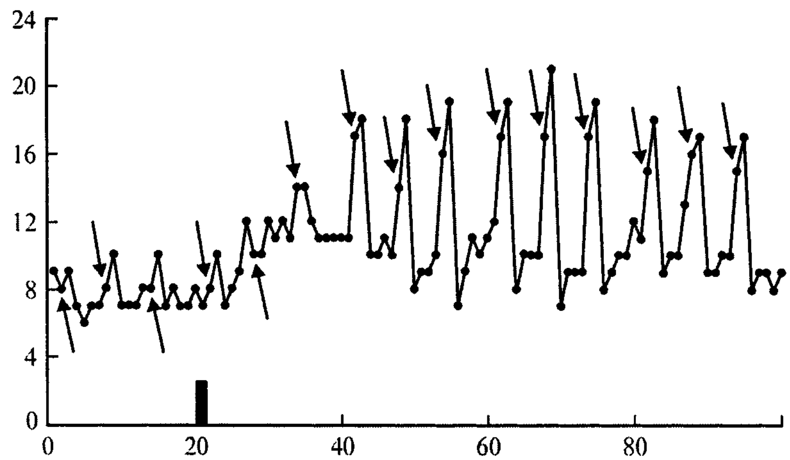

Fig. 5. Responses of an isolated PedA neuron to approximation to the PedA cluster after addition of 5-HTP to the chamber (final concentration 10^{-4} M) without flow. The neuron was moved as in the experiment described in Fig. 3. The black bar on the abscissa shows addition of 5-HTP to the chamber. For further details see caption to Fig. 3.

Changes in the Activity of Isolated Neurons Close to Pedal Ganglia in the Presence of Exogenous 5-HTP. 5-HTP added to the flow at a concentration of $(1–2) \cdot 10^{-4}$ M increased the activity frequency of isolated neurons to an extent similar to that observed previously [2]. Having started to increase after exposure to 3-HTP for 3–5 min, the frequency reached a peak at 15–20 min and then started to decrease regardless of whether the presence of 5-HTP in the chamber was maintained. The frequency increase induced by approximation to pedal ganglia also changed in the presence of 5-HTP, though the kinetics of changes were slower. The absolute increase in the frequency induced by approximation to pedal ganglia continued to increase as the baseline spike frequency reached a maximum 5-HTP-induced increase (three cases of four) (Fig. 4). In three other similar experiments, in which the flow was absent and 5-HTP was not washed out of the chamber, the nature of the response to 5-HTP was similar (Fig. 5).

DISCUSSION

Our experiments showed that isolated serotonin PedA neurons (body and proximal process), when approximated to the cellular cortex of the ganglion, experienced strong effects from local neuroactive factors which apparently had similar effects on the bodies of neurons located in this region of the CNS surface. The excitatory action was strongest in the region of the pedal ganglia containing the serotonin PedA cluster.

Two facts provide evidence supporting the view that the main excitatory substance was 5-HT. The first is that exogenous 5-HT, acting in concert with factors released from ganglia masked the actions of this endogenous factor on isolated neurons – partially at low concentrations and completely at a concentration of $5 \cdot 10^{-5}$ M. The second is that the excitatory effects of the pedal ganglia on isolated PedA neurons increased in the presence of 5-HTP which, as we have demonstrated previously, has no direct effects on PedA neurons, but acts only after conversion to 5-HT [10]. It should be noted at this point that although exogenous 5-HT in these experiments acted both on pedal ganglia and on isolated PedA neurons, the characteristic time differences between these actions allow the effects of additional 5-HT secretion from the PedA cluster to be distinguished from the effect of additional 5-HT synthesis by the isolated neuron itself.

According to our results, simultaneous somatic secretion by neurons of the PedA cluster maintains the 5-HT concentration at its surface at a level of 10^{-8}–10^{-7} M. This is sufficient for the involvement of serotonergic neurons in the process of self-amplifying excitation and for the effect 5-HT-ergic control of other neurons located in this region of the cellular cortex of the pedal ganglion.

Thus, our data support the previously formulated hypothesis that activation of behavior in serotonergic arousal includes a positive feedback mechanism [4]. Previously reported results [1, 2, 9, 10] and those obtained from the experiments reported here, including those using isolated 5-HT-ergic neurons, also allow the chain of events following the action of exogenous 5-HT to be interpreted: 1) arrival of 5-HT inside the neuron; 2) additional intracellular 5-HT production; 3) release of 5-HT, particularly from the cell body; 4) action of secreted 5-HT on excitatory somatic autoreceptors located close to the spike generation zone; 5) changes in electrical activity. Furthermore, our results show that 5-HT released from nerve cell bodies is active not only in the immediate perimembrane space, but also at a distance form the releasing neuron. This results in self-amplification of excitation within the cell cluster and cooperation of 5-HT neurons. It can be suggested that an analogous mechanism underlies the self-amplification of the activity of 5-HT neurons in *Clione* [4] and *Aplysia* [15], but not in tritons, in which 5-HT cells are atypically involved in a generator responsible for defensive behavior, i.e., active avoidance [14].

Our results point to tonic release of transmitter into the intercellular space by many PedA neurons simultaneously. The following hypothesis can be formulated: intersomatic excitatory interactions of neurons in the PedA cluster are so strong that activation of a few members of this cell group initiates a functionally significant process of self-excitation of the PedA cluster as a whole.

Occasional cases of hyperpolarization of isolated PedA neurons in the absence of external chemical influences on the pedal ganglia, as well as decreases in the spike frequency of isolated neurons when approximated to the PedA cluster in the presence of exogenous 5-HT, showed that the medial part of the pedal ganglia secretes not only 5-HT, but also other neuroactive factors – at least some substance with hyperpolarizing actions on PedA neurons. These factors may also mediate interneuronal interactions at the level of cell bodies.

The hypothesis of non-synaptic interneuronal interactions based on the integral actions of neurons within a local neuronal system has been addressed at the theoretical level [6, 7] and has been supported by experiments on unidentified neurons in *Clione* [12]. In the present report, we have provided the first direct experimental evidence for the involvement of this type of mechanism in controlling the electrical activity of neurons with known behavioral functions. The activity of such neurons can be regarded as shown to depend on the neurotransmitter apparatus and the cellular cortex of the ganglia. This significantly widens our knowledge of the nature of interneuronal interactions in clusters of nerve cells.

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