Review

Cellular mechanisms of behavioral plasticity in terrestrial snail

P.M. Balaban* 

Laboratory of Cellular Neurobiology of Learning, Institute of Higher Nervous Activity and Neurophysiology, 5A Butlerova street, Moscow 117865, Russian Federation

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Abstract

Functional organization of networks underlying withdrawal, feeding, and respiration in terrestrial gastropod snail Helix are described. Tracking the changes during non-associative and associative modifications of behavior, analysis of plasticity mechanisms in identified neurons involved in these networks allowed to formulate several conceptual principles which are not widely accepted. The review will present data underlying the following principles:

1. Command neuron concept can be applied only to all-or-none behavior.
2. Habituation is an active down-regulation process opposite to up-regulating sensitization. All long-term behavioral changes at least in part are associative.
3. Reinforcement is a motivational state mediated by neuromodulatory neurons and can be produced by activity of a single modulatory neuron.
4. Non-addressed (‘soft-wired’) neuromodulatory influences are necessary for acquisition of memory, while retention of memory depends mostly on ‘hard-wired’ local changes in synaptic connectivity.
5. Retrieval of declarative (sensory) and procedural (motor) memory involves different functional classes of neurons. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Learning; Serotonin; Reinforcement; Identified neurons; Command neurons; Neuromodulation

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* Tel.: +7-95-334-7810; fax: +1-617-687-3051.
E-mail address: balaban@ihna.msk.ru (P.M. Balaban).
1. Introduction

It is not necessary now to praise the usefulness of neurobiological investigations in invertebrates. A number of neuroscientists interested in cellular analysis of behavior are exploiting the unique properties of invertebrate nervous systems, relatively simple and stereotyped behavior. Investigations of cellular mechanisms of learning and memory in invertebrates became an important part of contemporary neuroscience.

Learning (as a part of behavior) is an emergent property of the nervous system. Pinsker [1] defined an emergent property as the one possessed by an entire system but not by its individual components. We consider the concept of emergence to be the main problem for anyone using reductionist strategy in his research. Since an emergent property is not possessed by the individual components, there is only one sequence to follow when analyzing underlying mechanisms. One should begin with characterization of the phenomenon as a whole and then isolate the components for analysis. Molluscs provide an extremely useful model in this respect taking into account that their behavior is relatively complex, and the nervous network is relatively accessible for the analysis. But is their behavioural repertoire relevant for the psychological problems? There have been a number of demonstrations of higher brain functions (including associative learning) in a variety of invertebrates including Helix [2–6]. Even a possibility for self-stimulation was shown in terrestrial snails by Balaban and Chase [7].

A detailed analysis of behavior is a necessary prerequisite of neurophysiological studies. Present work is a review (some results were published only in Russian) concerning the description of two types of memory in terrestrial snails (Helix sp.), and an investigation of the role of individual cells and neuromodulatory systems in learning. By analyzing the behavior and memory in snails, it was possible to distinguish declarative memory (which do not require any motor response to a certain stimuli, but can influence behavioral performance), and a procedural memory, which is manifested in changes in certain motor responses to a certain stimuli.

One of the most difficult problems in neurobiology is functional identification of neurons involved in network underlying certain behavior. Only primary sensory neurons and motor neurons can be easily identified due to their
morphological connections to receptors and muscles. All other neurons involved in behavior interact with one another, and detecting their contribution to behavior constitutes an independent task.

2. Functional organization of Helix nervous system

Using relatively simple nervous system of terrestrial snail Helix we tried to formulate criteria for the identification of behavioral function of neurons and to describe the networks involved in withdrawal, feeding, and respiration.

2.1. Morphological, electrophysiological, and behavioral criteria for functional identification of neurons

We used multiple criteria for functional identification of cells. In the nervous system of Helix there are about 20 000 neurons in 11 ganglia plus about 60–80 000 small uniform cells in procerebrum involved in olfaction [8]. Only several tens of them are individually identifiable. Identifiable cells, whose function was investigated, are numbered on the map (Fig. 1). Beside morphological and electrophysiological properties of the neuron we used for functional identification a responses to nerve and sensory stimulation in semi-intact preparations with preserved connections of the central nervous system (CNS) to the periphery (Fig. 2), ability to elicit contractions of effectors to intracellular activation of a single cell, comparison of dynamics of neuronal responses to sensory stimuli with dynamics of behavioral responses. Results of functional identification reflecting our knowledge of networks underlying several forms of behavior are summarized in this section.

2.1.1. The network underlying withdrawal behavior

Four groups of nerve cells participating in withdrawal behavior have been morphologically and functionally identified in Helix.

2.1.1.1. Sensory cells. The cells which we refer to as sensory had the following properties: the latency of their response to noxious stimuli evoking withdrawal reactions approached the time needed for impulse conduction from the stimulation site to the CNS; the receptive field of these neurons was limited; the amplitude of EPSPs in the interneurons had a linear relationship with the number of action potentials in the putative sensory cells; a spike response to the noxious (tactile) stimuli depended on the intensity of the stimulus,
was noted between these cells in during strong activation. No synaptic or electrical coupling behavior, but contractions of musculature can be seen of the motorneurons does not evoke goal-oriented beha-
vioral reaction, but contractions of musculature can be seen of the motorneurons does not evoke goal-oriented beha-

2.1.1.2. Motor neurons

Motor neurons. The most characteristic feature of motorneurons is that the spontaneous spiking activity correlates with the spontaneous movements of effectors innervated by these cells. These cells respond to all external stimuli evoking the behavioral withdrawal reactions. The response usually is represented by a spike discharge of a duration corresponding to the behavioral response, but not to the duration of stimulus. In isolated CNS these cells are usually rhythmically active, while in semi-intact preparations a strong synaptic modulation independent of the external stimuli is observed. Intracellular stimulation of one of the motorneurons does not evoke goal-oriented behavioral reaction, but contractions of musculature can be seen during strong activation. No synaptic or electrical coupling was noted between these cells in Helix. Synaptic input of the investigated motorneurons was synchronous to a large extent, but its effectiveness varied in different cells. Motorneurons in which a discharge was recorded during withdrawal reactions always receive excitatory synaptic input from tactile receptors.

2.1.1.3. Modulatory neurons

A group of serotonergic cells was identified in the pedal ganglia capable to modulate the synaptic input of the command cells (description follows) for withdrawal behavior. Extracellular or intracellular stimulation of these cells led to facilitation of spike responses in the command neurons for withdrawal behavior to noxious stimuli. Two components of facilitatory effects were found, one being efficient for the cells which are presynaptic in relation to the command cells for withdrawal behavior, and the second one being efficient for the command cells. Increase in spontaneous spiking frequency in the modulatory neurons during facilitation of responses in command neurons was seen, and this frequency was dependent on the level of satiety of the animal. Detailed description of these cells will be given in the following sections (Section 2.3).

2.1.1.4. Command neurons

Nine neurons in parietal and pleural ganglia were shown to trigger the withdrawal responses [10]. Their properties and role in the network underlying withdrawal appeared to be essential, and their description is given in Section 2.2.1.

2.2. Premotor withdrawal interneurons = command neurons

Nine individually identifiable giant cells (Fig. 1A, cells 1 and 2 in both pleural ganglia, cells 2 and 3 in both parietal ganglia, and cell Left Parietal #5-LPa5) with the following properties were described [10]: (1) spike discharge evoked intracellularly in any one of these cells elicited coordinated withdrawal reactions specific for a given cell; (2) spike response to a noxious stimuli always preceded or coincided the withdrawal reactions; (3) primary sensory mechanoreceptive neurons are monosynaptically connected to these cells; (4) spontaneous activity of the investigated interneurons had no correlation with the spontaneous (not evoked by external stimuli) movements of effectors; (5) mechanoreceptive, chemoreceptive, photoreceptive and thermoreceptive pathways converge on these cells; (6) intracellular activation of these cells in semiintact and isolated CNS preparation activates nerve cells presumed to be motor neurons; (7) intracellular hyperpolarization of one of these cells eliminates from the withdrawal reaction to a noxious stimulus a component similar to the component elicited by intracellular activation of the same cell.

The identified interneurons can be described as a premotor cells receiving convergent synaptic input, and capable of triggering the components of withdrawal behavior. Their function can be defined as ‘command’ in

Application of transmitters changed the pattern of activity in all recorded motorneurons synergically. Properties of the motorneurons suggest their functioning as a synergic groups of cells with a common input and functionally common output.

Fig. 2. Scheme of semi-intact preparation of terrestrial snail for intracellular recording, tactile, food and chemical stimulation of the lip chemoreceptors. Recording of movement on the preparation was performed with the aid of a pair of photo-diode and light-emitting diode.
accordance with the definition of Kupfermann and Weiss [11] if three proposed criteria are fulfilled. The cells in question respond to the presentation of a noxious tactile stimulus by a discharge which precedes or coincides the behavioral reaction (Fig. 3D; 'participation' criterion). Firing the neuron in its normal pattern releases the same or part of the behavioral response (Fig. 3A–C; 'sufficiency' criterion). The last, 'necessity' criterion is fulfilled in this nervous net in only a limited number of cases, because the pneumostome closure is mediated by several independent putative command neurons, the behavioral reaction being the net outcome of their activity. Thus the removal of one neuron abolishes only a part of the whole behavioral reaction. In some cases, however, contribution of one neuron to the whole withdrawal reaction can be singled out, and in these cases the necessity criterion is fulfilled. An example is presented in Fig. 3.

Three to four spikes induced intracellularly in cells LPa3 or RPa3 (left, right parietal) elicited small but distinct reaction of the pneumostome (Fig. 3A–C). Similar and even greater discharge can be evoked in these cells by a moderate tactile stimulation of the mantle. Unfortunately, the existence of a peripheral net in the mantle and the location of the pneumostome in this organ did not allow a test of the necessity criterion, because the central component is masked by the peripheral one. Tactile stimulation of the foot separated in the semi-intact preparation (Fig. 2) from the mantle evokes usually one–two spikes or subthreshold excitatory postsynaptic potentials (EPSPs) in the putative command neurons. In Fig. 4 examples of phasic responses in command neurons to moderate intensity tactile stimulation are shown. In the experiment shown in Fig. 3 we have increased the spike response in a putative command cell by a small constant depolarization (6 mV) up to three spikes (Fig. 3D), and after 30 min of rest the spike response was abolished by a constant 10 mV hyperpolarization (Fig. 3E). If one would compare the withdrawal reaction evoked by a similar tactile stimulation in Fig. 3D and E, absence of the first phase of reaction in Fig. 3D will be apparent. Intracellularly induced discharge of this neuron elicits the reaction similar to the abolished part of the whole reaction (Fig. 3A). Intracellular activation of command neurons never elicited any excitation in another command neurons, but effectively activated motorneurons of visceral ganglion involved in pneumostome closure (Fig. 3F). Properties of these cells allowed us to assign them a 'command function' in the network underlying withdrawal behavior. Formally, every neuron of this group can be called a command cell in accordance with the definition proposed by Kupfermann and Weiss [11] because they are necessary and sufficient for triggering a part of a coordinated behavioral acts.

Participation of the command neurons in withdrawal to temperature stimulation of the skin is shown in Fig. 5. Moderate local heating (a wire heated to 70 °C placed at 2 mm from the skin) of the skin elicited local contraction at the heating site (Fig. 5A) and a small quantity of spikes in the recorded command neuron. Strong heating (120 °C) elicited general withdrawal of all parts of the body and a strong response with spike frequency 10–20 Hz in the same neuron (Fig. 5B). All known command neurons are activated when generalized withdrawal is elicited.
the network underlying withdrawal behavior can be additionally characterized in experiments in which the dynamics of the behavioral response to an adequate stimuli is compared with the dynamics of a functionally similar responses of the same effector (pneumostome in our experiments) to the intracellularly induced spike discharge in the CNs [12]. The results of one of such experiment is presented in Fig. 6A. No sensitization was seen in number of spikes elicited by the regularly applied (10 s duration, 1 per min) fixed amplitude depolarizing current in the CNs, as well as in the amplitude of withdrawal reactions (pneumostome closures) induced by the discharge in CNs. Amplitude of the behavioral responses (pneumostome closures) recorded in the same experiment to a rhythmic tactile stimuli with same frequency underwent sensitization and subsequent habituation (Fig. 6A), as is normal for this form of behavior (for details see Refs. [13–15]). These results confirm the essential role of command neurons in the nervous net underlying withdrawal behavior. The decision to trigger the withdrawal reactions is represented in this net by a phasic spike discharge evoked in the specific command cells by converging sensory input [16].

It should be stressed that in majority of experiments the function of the investigated cells heavily depends on the whole neuronal system involved in regulation of a given behavior. The role of individual cells is restricted to a part of

Fig. 5. Response of a command neuron (lower trace) and movements of left and right parts of foot and mantle (traces from the top to bottom) to local heating of moderate intensity (A), and strong heating evoking generalized withdrawal (B).

Fig. 6. (A) Difference in dynamics of behavioral responses amplitude to a rhythmic tactile stimuli (closed triangles), and dynamics of same behavioral reaction amplitude to intracellularly induced discharges in the command neuron (closed circles); number of spikes induced in the command neurons is shown as open circles. All stimuli were delivered 1 min^-1. Initial response was taken as 100% (ordinate). (B) Correlation between amplitude of behavioral reaction (closed circles) and number of spikes in responses of a command neuron to a rhythmic tactile stimuli (open circles). Interstimulus interval 10 s. Initial response was taken as 100% (ordinate). (C) Dynamics of responses in sensory (closed circles) and modulatory (open circles) neurons involved in withdrawal behavior to similar tactile stimuli as in B. On the inset a schematic structure of the network underlying withdrawal is shown.
the behavioral act. In all cases, it is evident that the behavior of the animal is not controlled by individual command neurons, but by a synergically functioning conglomerate of cells with distinct functional roles.

A simplified schematic representation of the network underlying the withdrawal reactions of the snail is shown in Fig. 6 (inset). The command neurons are suggested to be a critical (‘decision’) link in this network of cells participating in withdrawal behavior. It must be noted that besides an input from command cells, motor neurons receive synaptic input directly from sensory cells, because the latency of the synaptic response to noxious stimuli in motor and interneurons is the same. This suggests, that the network can work without command cells, although its functioning would be different.

2.2.1. Who makes the decision?

In an attempt to characterize in the net underlying withdrawal reactions the link, which is responsible for making a decision, we investigated a correlation between responses in functionally different cells and the amplitude of a behavioral reaction. An example of such experiment is shown in Fig. 6B and C. It is evident that the activity in command neurons reflects the net output, because the dynamics of its spike discharge (number of spikes) closely follows the dynamics of behavioral reaction (Fig. 6B). From Fig. 6C it can be seen that the spike responses of sensory and modulatory cells do not follow the same pattern as the behavioral response. Response pattern of motoneurons closely resemble the pattern of responses of command neurons. Presented results, in full conformity with the previous data, suggest a critical role of the command neurons in the network underlying withdrawal reactions. These cells represent a last stage of integration of sensory information in the nervous system before initiation of the motor program.

It must be stressed that functionally the same behavioral events can take place without participation of the command cells, for example the pneumostome closure can be elicited by noxious stimuli or can occur spontaneously. In the latter case the command neurons do not participate in triggering this behavior. It is our opinion that the command neurons conforming to the definition of Kupfermann and Weiss [11] are ‘designed’ by the nature only for triggering the escape or withdrawal reactions to external noxious stimuli. In other types of behavior, participation of command neurons is questionable. Almost all described in the literature examples of a putative command neurons in invertebrates are a cells involved in escape, startle or withdrawal behaviors.

2.2.2. Command neuron versus command function

The concept of command appeals to a simple idea that some cells (or a group of cells) are superior to the others, and initiation of behavioral act is triggered by higher-order interneurons with the specialized responsibility for initiating behavior patterns. It was hypothesized that the initiating cells were ‘decision-making’ interneurons responsible for receiving the necessary sensory information for the given behavior and then firing to initiate or permit the expression of a behavioral response [17].

More than 60 years ago a proposal was made that there are individual neurons responsible for initiation of a complex behavioral acts [18,19]. It is essential to note that although this idea was first developed from work on the all-or-none escape responses of invertebrates, the conceptual framework was so simple and mechanistic that it was quickly adopted for more complex behaviors [20]. In many animals, including vertebrates, initiating cells were found in networks underlying different types of behavior. More than that, different versions of command function (triggering, gating, tonic or phasic modes of action, etc.) were described, and the term command neuron was often misused or abused by skeptics.

The most confusing quantity and variety of command cells was found in networks underlying rhythmical behaviors. In all such networks the command function represent an emergent property of the network, rather than being confined to a single higher order element [21]. On the contrary, in snails, insects and even fishes, individual premotor cells command an all-or-none escape reaction and no confusion arises in these cases.

The command concept came to be used for almost all animals, but many investigators soon found that it is not possible to use such a simple mechanistic explanation for all behaviors. An extensive analysis of the necessity and sufficiency paradigm (suggested as a definition for command cells by Kupfermann and Weiss [11]) was carried out on the Mauthner cells of teleost fishes [20]. The results showed that ablation of the Mauthner cell does not impair dramatically the escape behavior. The authors raised a question of the possible inadequacy of the command concept for neurobehavioral research.

Lerimer [22] came to a different conclusion in the paper devoted to an analysis of applicability of the command hypothesis to a system of interneurons underlying abdominal flexion and extension in crustaceans. In this work the author suggests that a typical abdominal positioning interneurons operate as groups of command elements (every one of which is not necessary for triggering a specific behavior) in a larger command system. In spite of their ‘diminished status’ (relatively to the status of command neurons which are necessary for triggering behavioral events), command elements occupy the key positions in this motor system.

We propose to distinguish these two experimental situations, and restrict the use of the term ‘command neurons’ only for those premotor cells involved in all-or-none behaviors. In all other cases the command function cannot be assigned to individual cells and is probably distributed in the network.

2.3. Neuromodulation of withdrawal behavior

Modulatory effects of some neurons and transmitters
neurons are readily identified in mollusks is the monoamine serotonin (5-HT). Serotonergic neurons have been considered in the literature [23–25], and one of the most likely modulators for withdrawal reactions in testing stimuli.

Fig. 7. Increase of a complex EPSP (up to a spike threshold level) evoked in withdrawal interneuron LPa3 by rhythmic (1/20 s) stimulation of a cutaneous pedal nerve after extracellular stimulation (arrow) of 5-HT-containing pedal cells (3 ms, 5 Hz, duration 5 s) in the interval between testing stimuli.

have been considered in the literature [23–25], and one of the most likely modulators for withdrawal reactions in mollusks is the monoamine serotonin (5-HT). Serotonergic neurons are readily identified in Helix with a glyoxylic fluorescence histochemical technique [26,27]. In the present section, modulatory effects of stimulation of the serotonergic cells on the responses and excitability of neurons involved in withdrawal behavior are described.

2.3.1. Localization and morphology of putative modulatory cells

We looked for putative modulatory neurons for withdrawal reactions by stimulating extracellularly the groups of identified cell somata via a suction electrode and comparing the responses to test stimulation of the nerve before and after the extracellular stimulation. A complex EPSPs elicited by test stimulation of nervus cutaneus pedalis secundus sinister were recorded in the premotor interneurons (command neurons) for withdrawal behavior: giant pleural and parietal cells LPI1, RPI1, LPa2, RPa2, LPa3, RPa3. All these cells are functionally similar (Section 2.2.1) [10], and bilaterally located neurons receive common synaptic input.

Stimulation of only one group of cells in the rostral part of the pedal ganglia (Fig. 1) was able to affect significantly the complex EPSP amplitude to the test stimuli in the withdrawal interneurons (Fig. 7). Complex EPSPs in pleural cells controlling tentacle and head withdrawal increased simultaneously with responses to the same stimulus in parietal cells controlling contraction of the middle part of the foot, mantle bolster, pneumostome. This modulatory effect was highly reproducible and lasted 2–5 min. After a period of rest, sensitization may be evoked again. Several sensitizing stimuli in a row (2–5 stimulations with interval 10 s were tested) led to increase of duration of sensitization. Sensitization was seen in all preparations in which it was tested. Complex EPSP amplitudes significantly increased after stimulation. The average level of increase was 64 ± 12% (mean ± SEM, 28 snails, 42 recordings in withdrawal interneurons, p < 0.001). No significant difference was found in responses of different withdrawal interneurons. In spite of evident effect we regard these experiments only as pilot ones because there always exist a possibility that the current spreads to non-serotonergic cells.

Therefore effects of individual cell stimulation were investigated.

Identification of individual cell as serotonergic was based mostly on morphological data and prewashing with 5,7-DiHT. In order to obtain selective vital staining of serotonergic cells, snails were injected 2 months previously with selective for serotonergic cells neurotoxin 5,7-DiHT twice with 1 day interval, 10 mg/kg of weight (details in Ref. [28]). It was shown that 2 months after the neurotoxin injection the synaptic connections and normal levels of 5-HT are restored in the serotonergic cells [29,30], whereas somata of the cells obtain for a life-time a permanent red-brown staining [28,30]. Comparison of pigment labeling by 5,6-DiHT or 5,7-DiHT and immunolabeling techniques established that all the pigment-labeled neurons show 5-HT immunoreactivity in Helix [31].

From earlier studies [27,28] it is known that cells located in the medio-rostral part of the pedal ganglia both on dorsal and ventral sides contain serotonin. Only serotonergic cells were found in the rostral zone of the pedal ganglia which was the most effective region to elicit facilitation of withdrawal responses. Each half-ganglion contains 30–40 serotonin-containing cells in the rostral part, and several small clusters on the ventral side. Our results suggest that modulating effects on withdrawal behavior neurons can be attributed to pedal serotonergic cells.

Using the method of retrograde transport of Co²⁺, we traced neurons sending axons from the pedal ganglia to the neuropile of the parietal ganglia. Staining via parieto-pleural connective consistently revealed the Pd4 serotonergic cell, 1–2 randomly observed small serotonergic cells in the rostral part of pedal ganglia, a couple of identifiable large non-serotonergic cells, and two groups of small non-serotonergic cells (Fig. 8A). Taking into account those processes of only Pd4 cell overlap with processes and putative synaptic region of the giant parietal (command) cells in neuropile of the parietal ganglia (Fig. 8), it is logical to assume that mainly the Pd4 cell exert the described contingent changes in amplitude of synaptic input of withdrawal parietal interneurons.

2.3.2. Sensory input of modulatory serotonergic cells

Responses of modulatory cells to skin stimulation were studied in 29 semi-intact preparations (58 cells recorded). Tactile stimulation of low intensity evoked a phasic synaptic and spike responses in the withdrawal interneurons, and no immediate reaction in any recorded serotonergic cells. Increase of stimulus intensity led to significant increases of spontaneous firing frequency in serotonergic cells for at least 15–20 s. It is essential to note that latency of input coming to serotonergic cells is greater than the latency of response in withdrawal interneurons, and the latency was not dependent on the place of stimulation.

Regular presentation of tactile stimuli usually resulted in facilitation of the second and third spike responses in a series in the withdrawal interneurons [13]. Facilitation of
spike responses in the withdrawal interneurones was accompanied by a tonic increase of background spike frequency in serotonergic cells (Fig. 9). The tonic increase (up to 2 min to a single 0.1 s stimulus) of spiking frequency following noxious stimulation was characteristic for all recorded 5-HT-containing cells. Responses to a negative chemostimulus (quinine, applied to the lip contained in a separated by thin wall chamber), known to elicit withdrawal reactions in Helix [14], were compared with reactions to stimulants positive for feeding behavior, e.g. carrot juice. Application to the lip of a drop of quinine (10^-2 M) elicited significant activation of serotonergic pedal cells as well as of the giant metacerebral cell which was used as a control (this cell is involved in feeding, and is known to respond to all types of chemical stimuli [15,80] for the effective strength of the stimulus (Fig. 10A). It was observed in all preparations that carrot juice applied onto the lip evoked a spike reaction in the metacerebral cell comparable to the response to quinine, but no significant change of activity to the carrot juice application was noted in the putative modulatory pedal neurons (Fig. 10B). In some cases (3 of 11 cells) a slight decrease of the baseline frequency was seen in these cells in response to stimulants positive for feeding behavior. This result points out to certain specificity of the investigated group of pedal serotonergic cells.

2.3.3. Interconnections between modulatory serotonergic cells

It was observed in experiments in which two or more serotonergic cells were recorded simultaneously (28 snails, 76 serotonergic neurons) that high frequency spontaneous firing in one or more cells is accompanied by correlated activity in simultaneously recorded serotonergic cells. When the rate of background firing was low, no strong correlation of activity was seen, but synchronous EPSPs were observed occasionally. We tried to investigate possible interconnections of serotonergic cells using strong intracellular activation and found that after a burst of intracellularly induced spikes, a ‘recruited’ activity is noted in the stimulated cells, and small positive deflections
are observed which resemble spikes in electrically connected neurons (Fig. 11B). Continuation of investigation in pairs (48 pairs of serotonergic cells) of pedal neurons revealed that in approximately 65% of cases (32 pairs) the serotonergic cells were electrically connected with an average coupling ratio about 0.1 (Fig. 11A). Weakness of connection allows the cells to be relatively independent when the firing background frequency is small, whereas when only several cells from a group receive strong excitatory input, other cells may be recruited.

2.4. Molecular biological approach to functional identification of neurons

Exploiting the idea that functionally similar neurons should have some common biochemical properties differing from all other neurons in the nervous system, we mechanically isolated the command neurons of parietal ganglia (LPa3 and RPa3) from 20 snails and used a combination of cDNA subtraction cloning and differential screening approaches in order to find some genes which are expressed specifically in these neurons. The hypothesis was that if we will be able to find such neuron-specific genes, the pattern of this gene expression would allow us to identify other members of the functional group which we were not able to find electrophysiologically.

2.4.1. Withdrawal command neuron gene

Using a combination of cDNA subtraction cloning and differential screening approaches we have isolated a two novel genes named Helix Command Specific #1 and 2 (HCS1 and HCS2) which were expressed predominantly in a subset of premotor withdrawal interneurons (command neurons for withdrawal [32,33]). Expression of HCS1 was observed only in two giant parietal neurons (LPa3 and RPa3) from about 100,000 of neurons in the snail central nervous system, while expression of the HCS2 was not so extremely specific and was observed in all four parietal command neurons, and several unidentified cells in other ganglia.

The predicted amino acid sequence of the HCS2 protein contains at the N-terminus a hydrophobic leader sequence and four putative neuropeptides, and at the C-terminus a perfect match to the consensus motif of the EF-hand family of the Ca\textsuperscript{2+}-binding proteins. All four predicted neuropeptides bear a C-terminal signature sequence Tyr-Pro-Arg-X (where X is Ile, Leu, Val or Pro), and three of them are likely to be amidated. Physiological action of three synthetic peptides corresponding to the predicted mature HCS2 peptides mimics fairly well the described action of parietal interneurones on follower motorneurons controlling pneumostome closure. In situ hybridization experiments demonstrated that the HCS2 gene is selectively expressed in the four parietal giant interneurons, as well as in just several small unidentified neurons. The onset of the HCS2 transcription during embryogenesis coincides temporally with the time point when the first withdrawal responses of the embryo to tactile stimulation appear (details in Ref. [33]).

2.4.2. Modulation of the HCS2 gene expression

We tested whether a decrease of synaptic activity by blocking polysynaptic connections by divalent ions and effects of well known modulator of withdrawal networks serotonin will change the expression pattern of HCS2. It was found that increase of Mg\textsuperscript{2+} concentration significantly decreased expression of HCS2 in many cases to zero [34]. Application of serotonin elicited expression in identifiable neural clusters in pleural, cerebral, and pedal ganglia.

2.4.2.1. Functional significance of HCS2-expressing cells.

No HCS2 expression was observed under normal conditions.
in the pleural ganglia. The functional role of pleural cells is presumed to be motor and sensory. Under serotonin and other influences a distinct cluster of HCS2-expressing cells in pleural ganglia was observed (Fig. 12). Based on the pattern of HCS2 expression, we performed electrophysiological experiments which demonstrated, that at least some cells located in the HCS2-expressing group are presynaptic to premotor parietal (command) interneurons which express HCS2. Double-label experiments are necessary to find out exactly whether the sensory or motor cells in this cluster express the HCS2. Thus, at least some of the cells with HCS2 expression in pleural ganglia may be also involved in withdrawal behavior, serving a sensory function in responses to noxious stimuli.

HCS2 expression in the pedal ganglia in normal conditions was represented in most cases by a single 50 μm identifiable cell located on the ventral surface of the left pedal ganglion in the medio-rostral area. Under serotonin application and in preparations maintained for 3 days in culture medium about 30 neurons expressed HCS2 (Table 1). It should be noted that rostral area of the pedal ganglia is occupied by serotonergic cells known to be involved in withdrawal behavior. Their functional role involves withdrawal behavior modulation (see Section 2.3), and it was shown that their participation is a prerequisite for successful associative learning involving noxious stimuli as a reinforcement [14]. Additional cells in the middle of the ganglia and those that cluster near the pedal–cerebral connective have not been described before, and their participation in behavior is unknown. Thus, the major part of cells of pedal ganglia showing HCS2 expression are also involved in withdrawal behavior.

HCS2 expression in cerebral ganglia under normal conditions was observed only in two symmetrical small clusters of cells. These clusters contained 6–8 extremely small non-identifiable cells of 5–8 μM soma diameter, and expression in these cells did not change under different conditions. Some of the parameters used in our experiments elicited the expression in two additional groups, one of which (lateral) consists of cells of unknown functional significance, while the medial cluster consists of molluscan insulin-containing cells described earlier. The

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<th>Parietal neurons</th>
<th>Pleural neurons</th>
<th>Pedal neurons</th>
<th>Cerebral neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control animals (n = 19)</td>
<td>74.4 ± 9.8</td>
<td>14.8 ± 4.6</td>
<td>18.3 ± 5.8</td>
<td>19.4 ± 6.1</td>
</tr>
<tr>
<td>B Tail cut 24 h (n = 13)</td>
<td>76.9 ± 9.5</td>
<td>20.7 ± 4.6</td>
<td>23.0 ± 6.3</td>
<td>38.8 ± 6.8</td>
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<tr>
<td>C 24 h at RT (n = 15)</td>
<td>63.2 ± 10.8</td>
<td>25.8 ± 8.9</td>
<td>17.6 ± 4.8</td>
<td>26.7 ± 8.8</td>
</tr>
<tr>
<td>D 24 h at RT + 5-HT (n = 20)</td>
<td>88.7 ± 4.6</td>
<td>34.3 ± 7.7</td>
<td>66.3 ± 7.7</td>
<td>35.8 ± 6.3</td>
</tr>
<tr>
<td>E 24 h at RT + Mg2+ (n = 19)</td>
<td>55.5 ± 9.8</td>
<td>8.8 ± 4.1</td>
<td>9.0 ± 2.8</td>
<td>13.1 ± 4.6</td>
</tr>
<tr>
<td>F 24 h at RT + 5-HT + anisomycin (n = 13)</td>
<td>88.5 ± 5.4</td>
<td>2.6 ± 1.6</td>
<td>43.8 ± 11.6</td>
<td>34.7 ± 9.4</td>
</tr>
<tr>
<td>G 24 h at RT + Mg2+ + 5-HT (n = 4)</td>
<td>81.3 ± 11.9</td>
<td>0.5 ± 0.5</td>
<td>19.0 ± 10.5</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>H 24 h at RT + Dopamine (n = 4)</td>
<td>87.5 ± 12.5</td>
<td>0.75 ± 0.7</td>
<td>28.2 ± 8.1</td>
<td>21.5 ± 11.4</td>
</tr>
<tr>
<td>I 3 days at RT (n = 10)</td>
<td>92.5 ± 3.8</td>
<td>64.6 ± 11.5</td>
<td>88.3 ± 7.1</td>
<td>85.1 ± 5.8</td>
</tr>
<tr>
<td>J 3 days at RT + Mg2+ (n = 12)</td>
<td>64.5 ± 8.9</td>
<td>19.0 ± 8.4</td>
<td>26.7 ± 8.6</td>
<td>24.1 ± 7.8</td>
</tr>
<tr>
<td>K 3 days at RT + Forskolin + Mg2+ (n = 4)</td>
<td>100.0</td>
<td>69.5 ± 12.7</td>
<td>91.7 ± 8.2</td>
<td>81.5 ± 10.6</td>
</tr>
<tr>
<td>L 3 days at RT + TG + Mg2+ (n = 4)</td>
<td>87.5 ± 4.7</td>
<td>44.0 ± 8.9</td>
<td>54.4 ± 10.2</td>
<td>52.5 ± 13.7</td>
</tr>
</tbody>
</table>

Fig. 12. Distribution of cells demonstrating expression of HCS2 under normal conditions (open circles, arrows), and after application of serotonin (closed circles). PC, procererebrum; MsC, mesocerebrum; MtC, metacerebrum; St, statocyst.
insulin-like-containing cells were described in several molluscan species. Responsiveness to human insulin was shown, and the genes homologous to human insulin have been described. Thus, it is possible to speculate that the HSC2-expressing insulin-containing cells may be involved in the CNS response to long-lasting noxious stimuli involving growth of damaged axons (tail cut) or to chemical stimuli that mimic noxious stimuli (serotonin, thapsigargin). Thus, this gene may be an important part of organization of withdrawal behavior.

Results obtained in our experiments give evidence that the decrease in the number of HCS2-expressing cells can be effectively elicited by a decrease in neural activity. On the contrary, activation of isolated CNS by serotonin application results in significant increases in the number of HCS2-expressing cells. This suggests a possibility for up-regulation of this particular gene expression by a transmitter specifically involved in regulating withdrawal behavior in mollusks. It should be noted that dopamine (shown to be involved in feeding in Helix) applications were not effective in up-regulating HCS2 expression, suggesting specificity for serotonin.

Induction of HCS2 expression evoked by serotonin did not depend on protein synthesis. It was shown in experiments with application of anisomycin or cycloheximide. Up-regulation of expression in all ganglia except the pleural ones was not different from control preparations which did not contain protein synthesis blockers, but was significantly greater than the expression under Mg\textsuperscript{2+} (Table 1). This result suggests independence of the up-regulating the HCS2 expression pathway of the novel protein synthesis.

Application of forskolin (cAMP activator, Table 1, row K) significantly increased the HCS2 expression under Mg\textsuperscript{2+} in all ganglia, which suggests that the cAMP, a well-known second messenger for serotonin effects, may be involved in up-regulation. Application of thapsigargin (eliciting release of Ca\textsuperscript{2+} from intracellular stores) also was effective under Mg\textsuperscript{2+} for all groups suggesting possible participation of Ca\textsuperscript{2+} in regulation of HCS2 expression.

Our results suggest that HCS2 pattern of expression can be down-regulated by decrease of synaptic activity in the nervous system, and up-regulated by external noxious inputs, application of neurotransmitters and second messengers known to be involved in withdrawal behavior, maintenance of isolated ganglia in culture medium. When up-regulated, the HCS2 expression appears at least in part in neurons involved in withdrawal behavior (Fig. 12).

Usefulness of the approach which utilizes gene expression as the marker for a functional network is still have to be proved, but the promises are evident.

2.5. The network underlying feeding

In feeding behavior of snails appetitive and consummatory phases are easily observed. Appetitive phase contains active locomotion in the direction of food, and a light touches by either pairs of tentacles. We consider the behavior after the contact of rhinophores with food as the consummatory phase, which contains lifting of the head, contact with food by lips, and a rhythmic buccal mass movements resulting in scraping the food.

Rhythmic movements of the buccal muscles were taken as a simple reproducible event representing the consummatory phase of a feeding behavior. Two groups of alternatively active motorneurons were found in buccal ganglia driven by unidentified central pattern generator cells (CPG). The motor program of the rhythmic scraping is highly stereotypic, and can be readily elicited in isolated buccal ganglia. The details of this motor program are not essential in the context of this review, therefore only the effective inputs will be described.

It was found that serotonin applications and activation of serotonin-containing cells can modulate the frequency of rhythmic activity of the buccal mass, decrease the threshold for triggering these rhythmic movements, but they do not trigger these movements [35]. Giant serotonergic cells in the cerebral ganglia receive positive feedback from the central motor program, thus making it possible to maintain the rhythmic movements. Application of dopamine (10\textsuperscript{-5} mol/l) consistently elicited rhythmical activity in quiescent preparations, and increased the amplitude (but not frequency) of ongoing activity. No dopaminergic fibers were found in the cerebro-buccal connectives which are the only pathways for control from the cerebral and suboesophageal ganglia. It suggests that triggering of feeding movements is accomplished either by dopaminergic cells located in the buccal ganglia or by dopaminergic input to these ganglia. Examination with the glyoxylic fluorescence histochemical technique (Salimova N., Sakharov D., unpublished) revealed a large quantity of afferent fibers containing dopamine. It was assumed that the feeding can be triggered by an excitation coming directly from the peripheral chemoreceptors. In experiments in a reduced preparation consisting only of a lip, buccal ganglia and buccal mass this assumption was confirmed. Application of sucrose to the lip readily evoked normal rhythmical movements in spite of the absence of control from the central ganglia [35] in such preparations.

No individual central neurons whose properties would allow us to assign them the command function were found. The best candidates for the command role are the central pattern generator cells, because they are certainly active during the behavior in question, and they are necessary and sufficient for its initiation and maintaining. Nevertheless, in the literature a CPG cells without any obvious reason usually are not considered to be a command neurons. It is our suggestion that the ‘rigid’ way of functioning of these cells, and a great dependence on functional state of the animal regulated non-synaptically underlie this neglect.

The functioning of the feeding network is quite different from functioning of the withdrawal reflex network. In the withdrawal reflex circuitry, the modulatory influence
suppresses or sensitizes the network output, but never triggers or prohibits it, while in the feeding network a release of dopamine can trigger the motor program (sufficiency criterion). The role of dopamine in triggering the feeding is confirmed by the fact that chemoreceptive information travels via a pathway containing a great number of dopamine-containing fibers (correlation criterion). We were not able to determine whether dopamine is necessary for triggering feeding, but it can be assumed indirectly based on our data. In general, the results suggest that command function is distributed between CPG cells and dopamine-containing cells in this network, because without a certain level of dopamine the CPG cells would not fire in a normal pattern, and the dopamine itself can trigger feeding movements only when the CPG neurons are active.

2.6. The network underlying respiration

The opening and closing of the pneumostome were taken as behavioral events representing respiration in pulmonate snail *Helix lucorum*. Two groups of cells with alternating spontaneous activity were identified in the visceral ganglia [127]. CPG cells were not identified, but their inhibitory action was recorded in motor neurons involved in pneumostome closure, and simultaneous excitation from the same source was observed in motor neurons involved in pneumostome opening (the first and necessary phase of inspiration). The frequency of this motor program activity is modulated by the level of oxygen in blood, and by the presence of noxious stimuli.

Which cells make a decision to act in such a simple circuit? Besides the possibility that we have not found some essential element of this circuit, there are two responses to this question. The first response is that the CPG cells are the command neurons that make the decision to open the pneumostome. The command function thus becomes an emergent property of the whole ‘hard-wired’ motor program, which can act without any ‘decisions’ rhythmically for an unlimited time in certain conditions. The second response is that in this circuit there are no command elements, and that the command function is distributed within the whole network. Such point of view totally undermines the command neuron concept and shows its limitations.

2.7. Behavioral choice and decision making

A new major problem appears if we assume that the command function is distributed in the ‘polymorphic network’, as is proposed by Getting and Dekin [36]. Which cells make a decision for the whole network? The decision can be represented by changes in the combinations of higher order inputs that impinge on the network (the ‘orchestration’ hypothesis: [37]), but the available evidence shows that besides changes in inputs (if any), a significant changes synergically occur in the whole network, and the possibility that individual cells can direct such complex changes in different parts of the brain is very low.

An extremely simple and intriguing solution is proposed by Sakharov [38]. For almost every behavioral act (state) in invertebrates there are reciprocally acting transmitters (or a set of transmitters). Reciprocal (antagonistic) control is shown at the behavioral, neuronal and membrane channel levels in invertebrates. The output is thereby controlled by the concentration of transmitters (neuropeptides included) that are released. It is well known that the release is not restricted to the synaptic cleft, so the message can influence all members of the network, including cells which are not synaptically connected. This hypothesis includes as a part the command neuron and command function concepts, because individual cells release a particular transmitter (or a set of transmitters), as well as a group of command cells was shown to have a common transmitter (FMRFα), express same gene. Thus, the particular behavior is triggered at a certain concentration of a particular transmitter (or a set of transmitters). The command neuron concept in its original form [11] becomes a part of this hypothesis for a case when individual cells can trigger the behavioral act.

It must be noted that in the all-or-one escape reflexes a maximal speed is necessary, and classical synaptic control is used because it is faster, but when the whole network must be reconstructed for the needs of a new behavioral state, the non-addressed (non-synaptic) release of transmitters may be more relevant.

In this section the following conclusions can be made:

1. The command neuron concept can be applied in full extent to an all-or-none escape, startle or withdrawal behaviors. Command function may be distributed between several elements of the network underlying rhythmic behavior.
2. Effective control of the different parts of the network can be achieved by non-synaptic release of transmitter rather than by activation of an individual command cells.
3. The decision making in the nervous net can be attributed to one cell only as an exception (escape reactions). In most cases it is an emergent property of the network, and the command neuron concept is not applicable for analysis of all behaviors.

3. Non-associative forms of behavioral plasticity

Usually, two simplest forms of behavioral plasticity—habituation (negative learning) and sensitization (facilitation, dehabituation, etc.) are considered as independent ones. In spite of the existence of independent mechanisms underlying habituation and sensitization, we will consider them as a cooperative system.

Most of our data concerning simple forms of behavioral plasticity conforms to the dual-process theory of habituation delineated in 1970 by Groves and Thompson [39,40]. The
main standpoint of the theory implicates that the amplitude of behavioral response, elicited by repeated stimuli, is the net outcome of two independent processes: habituation and sensitization. Authors postulate that each stimulus causes two effects: (a) elicits the behavioral response via the short and direct pathway 'stimulus–motor response', and (b) changes the 'state' of the nervous system (general level of excitation, arousal, etc.). Repetition of the stimulus elicits habituation in the direct pathway, and decrease of the effect takes place to the second stimulus in a series. According to this theory, habituation: develops exponentially and comes to an asymptote, depends directly on the stimulus frequency and inversely on the stimulus intensity, may spontaneously disappear on cessation of stimulation, progressively increases during repeated series of stimuli, leads to generalization of the response to test-stimulus. Effective stimulus elicits increase (sensitization) of the response amplitude to the following stimuli. Sensitization occurs not in the direct pathway, but in neural systems responsible for the state of synaptic connections in the direct pathway; during application of effective repeated stimuli the sensitization at first increases, then decreases to zero; at small intensities of stimuli sensitization may not be seen at all; sensitization underlies generalization of responses to the test stimulus; repeated application of a strong stimuli may lead to appearance of sensitization manifested as response to time; dehabituation elicited by extrastimulus is connected with the sensitization. According to this theory, habituation and sensitization may develop independently, but interact for creation the nervous net output signal [39–41].

In our experiments in terrestrial snails it was found that the repeated adequate stimuli of small intensity elicit quick habituation. With increase of stimulus intensity, the sensitization shows up and habituation becomes slower. By interaction of these processes most part of parametric characteristics of habituation [42], which were readily observed in snail, may be explained. Parameters of habituation and sensitization in terrestrial snails do not differ significantly from those in other animals, and we will not describe in detail those experiments. Instead, we will try to analyze common and differing properties of these two processes, their possible mechanisms. The main unsolved question is, as it is seen to us, a possibility to consider the habituation as an active process, similar to sensitization.

3.1. Habituation as active process

There exist two possibilities to describe a decrease of behavioral response amplitude. First is a decrease of the signal in the nervous network due to natural (without active influence) decrease of either transmitter release in the synapses, or decrease of responsibility (receptor desensitization) of neurons involved in the given network underlying the behavioral response. In fact on this is stopped the progress of analysis of habituation mechanisms in the literature concerning molluscs after it was explicitly shown that the number of quanta of transmitter released from a presynapse decreases correspondingly during decrease of the amplitude of excitatory postsynaptic potential (EPSP) [25,43]. The second explanation may be of similar importance: decrease of synaptic effectiveness is not a result of waning of a signal due to repeated presentations, but is a result of active inhibitory influence on molecular mechanisms of transmitter release or postsynaptic membrane.

If we will assume existence of active process during habituation, which at least in part is responsible for decrease of behavioral response, the whole logic of events becomes equal for increase and decrease of the response. Summary net output depends in this case on the strength of the stimulus which can or cannot activate sensitizing processes, and activation of inhibitory processes which control habituation.

Confirmation of the hypothesis concerning active inhibition was found during analysis of properties of neurons involved in the withdrawal responses in terrestrial snails.

3.2. Neural correlates of habituation and sensitization

Extremely important for the analysis of habituation in snails are the results obtained by Elekes and Nassel [44] describing that investigated in our work command neurons for the withdrawal behavior contain neuropeptide FMRF-amide (Phe-Met-Arg-Phe-NH₂), which is a biologically active compound and is shown to suppress the amplitude of central synapses in *Aplysia* [45], and in terrestrial snail *Helix* [46]. In order to check whether the spiking activity in the command neurons releasing FMRFamide decrease the synaptic input to the same or adjacent cells, we used as a test a repeated stimulation of intestinal nerve with large intervals. The test stimuli evoked complex EPSPs in the command neurons releasing FMRFamide decrease the synaptic input to the same or adjacent cells, large intervals between test stimuli (10–20 min) were necessary to decrease the habituation to possible minimum. After stabilization of responses to test stimuli at a certain level, strong intracellular activation of one of the recorded command neurons was performed. Changes in EPSPs amplitude were monitored in a simultaneously recorded command neuron which received in parallel the same synaptic input, because calcium-dependent potassium current evoked by intracellular depolarization changed the resistance and decreased the EPSP amplitude in the stimulated neuron. Obtained results (Fig. 13) [47] demonstrate that synaptic input in a command neuron was depressed by intracellular stimulation of another command neuron, presumably by release of FMRFamide. It should be noted that no synaptic interconnections were observed between the command neurons for withdrawal (Section 2.2), and they are supposed to function in parallel. Branching of neurites in parietal command neurons for withdrawal occurs in the same neuropile which creates the basis for non-addressed influence of released FMRFamide
on synapses of other cells, and own synaptic input (Fig. 13, inset).

Final target of FMRFamide in molluscan neurons is shown to be the serotonin-sensitive potassium channels which are activated by serotonin and inactivated by FMRFamide [45]. It is essential to note that the number of FMRFamide-containing neurons in snail nervous system is even bigger that the number of cells containing serotonin. It creates the basis for a possibility to change the behavior of the animal. Thus, it can be assumed that in any withdrawal behavioral act in snail these two independent transmitter systems compete. Serotonergic system is switched on by the noxious stimuli, increasing the net outcome, while the second system is switched on only when noxious stimuli excite the command neurons, and leads to inhibition of the net outcome. Dynamic balance of these two systems determines the network output and the amplitude of behavioral response.

The assumed role of non-addressed FMRFamide release in behavioral habituation does not conform to well known data concerning selectivity of habituation. It should be specially noted that the absolute value of FMRFamide influence is very small, about 15–20% (3), while during selective habituation decrease occurs in the scale up to 90%, and is surely dependent on other mechanisms as well. Besides, the described mechanism of habituation is not opposing the presynaptic habituation evoked by other mechanisms (transmitter depletion, etc.), but is an addition to them.

3.3. Inconsistencies in results concerning habituation

From our point of view, in published results and concepts concerning habituation very notable contradiction exists, which cannot be explained by habituation as a decrease of accessible for release transmitter in a synapse. The contradiction may be easily seen when one considers the general biologic rule of use and disuse of organs [48,49]. According to this rule, constant usage of an organ (brain) improves its functioning, strengthens connections, while when we consider habituation which implies repeated usage, the main result is use-dependent weakening of connections in the network. It is interesting to note that morphological investigations of mechanisms underlying habituation in marine mollusk showed the long-term morphologically observable changes [50]. In this work authors were able to analyze the quantity of synaptic contacts between sensory and motor neurons of gill-withdrawal reflex during long-term changes in the reflex. It was shown that during long-term increase of withdrawal the quantity of vesicles ready for release and quantity of synaptic contacts between sensory and motor cells become correspondingly greater, and underlies the behavioral increase of reflex amplitude. In this case the organ is used, and the performance improved. While in the case of habituation where all the procedure except the strength of stimuli is the same, authors have found decrease in the quantity of synapses and quantity of vesicles ready for release. In a series of papers (reviewed in Ref. [51]), a conceptually important fact is shown: during long-term habituation synapse is still preserved morphologically, but is not effective in the network. These results contradict to the rule of use and disuse of organs, and may be explained only with the aid of mechanism of active inhibition of the synapses in question. Our results concerning recurrent inhibition as a result of repeated stimuli, were obtained in terrestrial snails [47] and demonstrate a mechanism for habituation which acts as an additional mechanism together with decrease of synaptic content, and conforms to the rule of use of organs: the inhibiting pathway is improved, and as a result of this improvement the changes in presynapse occur. Active inhibition allows to explain the immediate restoration of synaptic effectiveness after extrastimulus.

In numerous studies devoted to habituation, the question of restoration of habituated response due to decrease of stimulus intensity is considered as a separate problem. Originally such data were obtained by Sokolov [52] during analysis of orienting reflexes, and were discussed in detail in literature concerning mechanisms of habituation [53,54]. If increase in behavioral response due to increase in stimulus intensity is not surprising, because the additional sensitizing neurons may be involved, the restoration of habituated response elicited by decrease in stimulus intensity suggests existence of neural system which reacts to a novelty. Our results concerning recurrent inhibition of the network by effector part of a system also cannot explain the response to decrease in stimulus intensity.

3.4. Postsynaptic induction of synaptic plasticity in snails

Involvement of postsynaptic membrane in regulation of synaptic effectiveness was shown to be different in mammals and invertebrates. Long-term potentiation (LTP) of synaptic
transmission in the mammalian hippocampus results from concomitant pre- and postsynaptic activation [55] in a fashion similar to that predicted by Hebb [56]. Moreover, postsynaptic injections of depolarizing pulses without presynaptic stimulation can induce LTP-like changes of EPSPs, so-called intracellular potentiation (ICP), as demonstrated by Kuhnt and collaborators for hippocampal CA1 field [57] and reproduced for entorhinal [58] and visual [59] mammalian cortices. Evidences were obtained that hippocampal ICP shares common mechanisms with LTP induced by afferent tetanization [60]. Long-term facilitation of synaptic transmission in mollusks is believed to be induced mostly by presynaptic mechanisms. Synaptic facilitation in mollusks was shown to be induced by presynaptic activation, and the postsynaptic activation was shown to be neither sufficient nor necessary for potentiation of synaptic transmission [61].

These considerable differences suggested the existence of two principally different types of memory mechanisms for mammalian and molluscan LTP. However, LTP regulated by the postsynaptic voltage and depending upon activity, and the postsynaptic activation was shown to be induced by presynaptic tetanization [65]. A short-term (10–20 min) phase of synaptic facilitation induced by intracellular tetanization (IT) of identified snail central neurons [64]. A complex excitatory postsynaptic potentials (EPSPs) were simultaneously recorded from two functionally and morphologically similar command neurons for withdrawal (LPa3 and RPa3) receiving common synaptic input, one of which served as a control. Two phases of intracellularly induced potentiation (ICP) of synaptic responses were observed: a high-amplitude short-term phase lasting 10–20 min and a long-term phase lasting more than an hour. The mean EPSP amplitude measured 10 min after tetanization was 41.2 ± 4.9% larger than in the non-tetanized control neurons, and 50 min after tetanization the amplitude was 7.9 ± 3.1% larger. Only short-lasting (5–10 min) changes in passive membrane characteristics (posttetanic hyperpolarization and resistance decrease) were observed in postsynaptic neuron. Intracellular pressure injection of Ca\(^{2+}\) in postsynaptic neuron elicited increase of synaptic efficiency, while injection of Ca\(^{2+}\) chelators abolished effect of intracellular tetanization [65]. A short-term (10–20 min) phase of synaptic facilitation induced by intracellular tetanization was stable in all experiments, while a long-term phase (lasting 40–50 min and more) was observed only in a part of experiments (42%). Postsynaptic induction does not exclude presynaptic involvement in the ICP maintenance. Accordingly, the observed transient postsynaptic changes cannot explain ICP maintenance by themselves. We conclude that potentiation of synaptic responses following the IT in molluscan cells occurs with participation of postsynaptic mechanisms.

3.5. Long-term sensitization or declarative (associative) memory?

Environmental (contextual) conditioning is a form of learning in which the contingency between the reinforcing stimulus and environmental properties is set. Memory deriving from such associations can be named declarative (iconic, sensory) because no specific behavioral response is performed to the presentation of a specific context. Presence of declarative memory is manifested in changes of behavioral responses to a certain stimuli in two different contexts. In many cases the contextual memory is described as a long-term sensitization because it appears after some strong stimuli (for instance, electric shocks) without any apparent association with experimenter-controlled stimuli.

3.5.1. Contextual conditioning in snails

For investigation of environmental conditioning, responses to noxious stimuli in two different contexts were chosen for comparison. In the experimental set-up, the snail was tethered by its shell in a manner allowing it to crawl on a ball that rotated freely in a 0.01% solution of NaCl (Fig. 14A). The ball was laced with bare stainless steel wire to complete an electrical circuit between the animal’s foot and a carbon electrode placed in the water. Electric shock was delivered using a 1–10 mA, 0.5 s current through a macroelectrode applied manually to the dorsal surface of the snail’s foot. Punctate mechanical stimuli were applied with calibrated von Frey hairs permitting delivery of pressures ranging from 6 to 68 g/mm\(^2\).

In the first series of experiments each snail of the two groups was exposed for 20 min daily to the experimental set-up. All animals were tested by applying tactile stimuli in the experimental set-up and on the glass lid of terrarium in which animals were kept between sessions. Only snails from the experimental group received two electrical shocks per day for 5 days. No tactile stimulation was applied during the shock sessions. Three days after completion of sensitizing treatment (animals were fed during 3 days periods of rest), the responsiveness to the same tactile stimuli was compared in control and experimental groups of snails. An experimenter blind to the experimental histories of animals applied the tactile stimulus to the skin of the foot, and measured the withdrawal amplitude in percents of the maximal withdrawal taken as 100%. Testing was performed in the experimental set-up, and in the non-reinforced environment: on the glass lid of terrarium in which animals were kept between sessions. To reduce possible effects of recent handling, the test was administered no sooner than 5 min after the subjects had been placed in the environment. Only actively moving animals were tested. Five tests per day for 3 days were scored for each animal. No shocks were...
delivered during the test sessions. Results are presented in
Fig. 14B.

Before noxious reinforcement, no significant difference
in amplitudes of tentacle withdrawal to the testing tactile
stimuli in all groups of snails was observed (ANOVA was
used). Three days after a 5 days session during which
experimental snails received two shocks per day, testing of
responsiveness performed in the set-up used for sensitizing
revealed a significant (Mann–Whitney, two-tailed,
\( n = 9 \) snails, \( p < 0.001 \)) increase of the median response ampli-
tude in sensitized animals (Fig. 14B). The difference
between control groups in different contexts was not
significant (Fig. 14B). The amplitude of withdrawal was
significantly greater in shocked snail in the context
previously paired with the shock.

Our next step was to compare responses of control and
shocked snails in another environment. Results obtained
with the same animals tested on the glass lid of the terrarium
before and 3 days after (right two panels) the shock session.
(C) Mean amplitude of tentacle withdrawal reactions to test tactile stimulation of skin in normal (filled columns) and 5,7-DiHT-injected after sensitization
(open columns) groups of snails before and after the shock. Timing of shock and 5,7-DiHT injections is marked (arrows). (D) Mean amplitude of tentacle withdrawal reactions to test tactile stimulation of skin in two groups of sensitized snails in same environment (ball) differing only by acidity of water.
Responses of the same snails were scored before and after the shock session on the ball in both contexts before and 3 days after (right two panels) the shock
session. One group was shocked in set-up with normal pH (closed columns), another group in acid pH (open columns). Note that the snails demonstrate
increased responsiveness in both contexts, but significantly greater responses in a context in which were shocked.

3.5.1.1. Effect of 5,7-DHT injection. There is a growing
body of experimental data implicating serotonin in a wide
range of memory processes in mollusks [25], as well as in
vertebrates [66]. Investigations of a specific role of 5-HT in
sensitization and in cellular mechanism of classical
conditioning [67,68] suggest the role of 5-HT in acquisition
of conditioning.

One of the approaches which may be employed for
investigation of the 5-HT role is the selective ablation of the
serotonergic neurons. Both 5,6-DHT and 5,7-DHT (5,7-
dihydroxytryptamine) are known to be sequestered selec-
tively within serotonergic neurons by a high affinity uptake
system [128]. The toxin is oxidized intracellularly produ-
cing free radicals which ablate serotonergic terminals both
in vertebrates [129] and invertebrates [30,131,134].

It was shown previously in Helix that after the 5,7-DHT
treatment (ablation the serotonergic neurons) both the
sensitization of the withdrawal reaction and the associative
aversive conditioning are impaired [14]. The feeding
behavior of 5,7-DHT-injected intact animals was visually
normal, as were also the electrophysiological responses of the investigated neurons to single feeding and noxious stimuli. This is indicative of the fact that the absence of associative learning of this type is not due to changes of responsiveness of the neurons taking part in the feeding and aversive behavior, respectively.

The 5,7-DHT treatment after elaboration of aversive conditioning in Helix does not impair the conditioned responses [14]. This result suggests that the 5-HT-containing cells participate in associative learning during consolidation phase of the conditioned reflex (example of the procedural learning), but are not necessary during its reproduction.

We tested a possibility of the involvement of 5-HT-containing neurons, which modulate the network underlying avoidance responses [69,70], in contextual conditioning. Injection of 5,7-DHT led to disappearance of effects of training (Fig. 14C). Only responses of vehicle-injected sensitized snails differed significantly from responses of snails from other groups in both environments. Difference between vehicle-injected sensitized snails, and 5,7-DHT-injected snails also was significant (M.W., \( p < 0.001 \), two-tailed). This result suggests that 5-HT-ergic neurons are necessary for reproduction and/or maintenance of contextual conditioning.

### 3.5.1.2 Context with one cue different.

In the next series of experiments we tested snails in two contexts differing in only one feature. The only difference in the environment in which two groups of snails received sensitizing shocks for 8 days was the acidity of the water in which the ball they walked on was floating (Fig. 14A). Acidity of water in the set-up A was normal, while in the set-up B a citric acid was added. All naive snails sensed this difference, and never tried to make radular rasps (feeding) on the ball in condition B while such rasps were characteristic for condition A. One group of nine snails was sensitized (electric shocks) in the set-up A with normal water, while another group was sensitized in the set-up B with acidic water. Testing of each snail was carried out in both contexts before and after 8 days of sensitization training. Each response to tactile stimulation in one context (normal water) was compared to responses of the same animal in another context (water with citric acid).

Pooled responses of all snails to test stimuli before and after sensitization are shown in Fig. 14D. The difference in responses between these two groups was not significant before sensitization (Mann–Whitney, \( n = 9.9 \), \( p < 0.2 \), two-tailed), but became highly significant when tested 3–5 days after sensitization training (M.W., \( n = 8.8 \), \( p < 0.001 \), two-tailed). Responses of snails shocked in context B increased in this context relative the responses in context A. Similarly, the responses of animals, shocked in context A increased in this context. This outcome is consistent with the view that the animals had learned in which context they received shocks. It is essential to note that the snails need at least 5–8 days to learn the difference in the two contexts, and this differentiation lasts at least 2 weeks (snails were not tested at longer periods).

These results provide a demonstration in terrestrial snails of the associative nature of a phenomenon known in the literature in invertebrates as the long-term sensitization. In our experiments we used the 'non-associative' procedure similar to that used in Aplysia for elaboration of the long-term sensitization [51]. Presented data suggest that presentation of sensitizing stimuli inevitably elicits appearance of an associative long-term sensitization that can be observed only in a certain context. In fact, this associative long-term sensitization is equivalent to contextual (environmental) conditioning.

The long-term changes observed in withdrawal behavior of Helix after a non-contingent application of noxious but non-traumatic stimuli can be due to non-associative activity-dependent neuromodulation or associative (conditioned) enhancement of responsiveness non-specific for the testing stimuli, but specific to the environmental cues. We cannot exclude the non-associative component that obviously exists, and can be found in isolated preparations from sensitized animals [25], but shall concentrate the discussion on facts proving participation of associative processes in long-term enhancement of withdrawal responses.

The starting point of our investigation was a theoretical analysis of what happens during sensitization, what is the behavioral goal (adaptive significance) of an increase of responses to all stimuli. After a noxious stimulus all reactions of the animal to external stimuli are enhanced, and adaptive significance of the enhancement is obvious: to be ready for the next stimulus, that can be more damaging. But what happens in the long run? Is it possible to enhance non-selectively all responses to all potentially dangerous stimuli? If the animal can be non-associatively sensitized for several weeks [71,133], then adaptive value of sensitization will be reduced to zero, because appearance of a new type of noxious stimulus in different environment would be unnoticed in sensitized animal. Quite different situation would be if we assume that in the long run (weeks) the animal develops association between the sensitizing stimulus and environment. It is essential to note that no association is formed between testing and sensitizing stimuli applied non-contiguously, so one can speak about absence of association between experimentator-applied stimuli, but the long-term behavioral changes are selective and association is formed between environment and sensitizing stimuli. In our experiments we used a non-traumatic sensitizing stimuli and found several properties which can be due to associative nature of observed behavioral changes in terrestrial snail: (1) long-term changes in behavior appear not after a single strong stimulation, but after several days of stimulation; (2) responsiveness of the sensitized snails in different environments was maximal in the environment in which the snail was sensitized; (3) changing of only one environmental cue
changes the behavior. We can speculate that each noxious stimulus elicits two parallel chains of events. One is a non-associative change of responsiveness mediated by neuromodulators, largely dependent on intensity of the stimulus and lasting 1–2 days, i.e. the average time of proteins turnover. Independent events are triggered in parallel in the network by the same stimuli, and may be the same neuromodulators are involved, but these changes are dependent not only on the strength of stimuli, but also on contingency of sensitizing stimuli on environment, number of replications of similar situations.

4. Associative forms of behavioral plasticity

At the present time there is no doubt that associative modifications of behavior may be elaborated in gastropods. History of attempts of elaboration of conditioned response in snails starts with experiments of Thompson [72] in pond snail Physa gyrina. He associated tactile stimulation of foot with food presentation, and after 250 paired trials in 2 days the tactile CS elicited feeding responses in 39.6% of cases compared to 3.3% before conditioning. Unfortunately, no controls for associativity were performed.

In the end of 60-es, 70-es a bunch of papers concerning learning appeared [73–78]. But in all these studies either there were no necessary controls, or the non-associative changes were observed which were referred to as sensitization.

The first work that indubitably showed the associativity of behavioral changes after training was performed in a carnivorous marine mollusk Pleurobranchaea californica [135]. In this work the aversion to food was elaborated in the snails. In a control group which received similar quantity of randomized stimuli a certain level of sensitization was observed, but the changes in behavior in animals which received paired trials were significantly different. Food-aversion was elaborated in a slug Limax maximus by pairing food with toxic carbon dioxide. A long-term association of food with aversion was shown [79].

The first paper concerning learning in terrestrial snail Helix was carried out in 1973–1974, and published by Litvinov et al. [3]. Later on a detailed investigation of different forms of learning was made including instrumental conditioning, food-aversion conditioning, conditioned feeding to a noxious stimulus, etc. (for review see Refs. [80,81]). For neurophysiological experiments the food-aversion response was chosen as a simple and highly reproducible even in a dissected snail preparation.

4.1. Neuronal correlates of food-aversion learning in withdrawal interneurons

It is essential to study neuronal mechanisms of behavioral plasticity in cells the functional significance of which is known, and the best candidates in Helix, besides primary sensory neurons and motoneurons, are the withdrawal command neurons (CNs, Section 2.2) defined as both necessary and sufficient for the initiation of a given behavior.

Aversive learning is one of the most suitable paradigms for investigation, because it usually concerns two competitive behavioral acts, one of which changes dramatically due to pairing of two stimuli. Any modification of avoidance behavior must be readily detected in the CNs. We recorded changes of responsiveness after food-aversion learning in the giant pleural cells triggering head withdrawal, and in four giant parietal cells triggering the elements of withdrawal behavior: pneumostome closure and contractions of foot musculature.

A unique pair of bilaterally symmetrical giant serotonergic neurons were described in the cerebral ganglion of a number of gastropod mollusks. The metacerebral giant cells have been found to modulate activity of the motor neurons in the buccal ganglia involved in regular movements underlying food scraping by the snail. Taking into account the described chemosensory input of the cerebral cells, and participation in feeding behavior [35,82] we recorded in these cells the changes in responses to food before and after the learning procedure.

4.1.1. Changes in responses of neurons to food presentation after conditioning

At least 3–5 days before the experimental session, the snails were deprived of food. Neurophysiological experiments were carried out in semi-intact preparation modified in such a way that the skin encircling the mouth and base of the tentacles was exposed to the application of food stimuli (a drop of carrot juice) with minimal damage (Fig. 2).

The primary purpose of behavioral experiments is to generate rapid and obvious modification in motor responses, reproducible in neurophysiological preparation, and to demonstrate specificity of elaborated behavioral modifications for the pairing of conditioned and unconditioned stimuli. The withdrawal reaction as an unconditioned response meets these criteria, and the underlying neuronal circuitry is investigated in Helix (Section 2). We reinforced the food presentation by a strong electric shock that evoked generalized withdrawal reaction of the snail.

After 5–15 paired presentations of food and electric stimulation in one experimental day (session) at 10–15 min intervals, the snail exhibited withdrawal reaction to the presentation of food (usually carrot) used in the experiments. Five or more successive aversive reactions to food in the session were taken as a criterion for the conditioned reflex development. Testing of responsiveness to food stimuli was usually carried out 30–60 min after the last reinforcement or on the next day. From 27 animals 24 snails exhibited aversion reaction to food (not contacting the food during 2 min was considered as aversive response for a hungry snail) after the learning procedure in 78% of cases, while three snails were inactive after the learning session,
and died in 1–2 days. The snails receiving random presentations of food and shock exhibited aversion response in 18% of cases. The difference between groups was highly significant.

It is essential to note that the snails were fed only by cabbage between the experimental sessions and during an experimental session the feeding behavior was easily evoked by cabbage presentation. Ten (or in some experiments up to 25) food presentations and electric shocks presented at random evoked no apparent changes in feeding behavior in 18 animals (one snail was inactive after random stimuli).

In the semi-intact preparations made from the trained animals exhibiting learning, the responses to food (juice) application to the lip in the metacerebral cells involved in feeding were not significantly different from the animals subjected to random presentation of food and electric shock. On the contrary, in response to food the withdrawal interneurons displayed previously absent spike responses to food in 82% of cases (22 preparations), while in the random group only in 17% of trials. Thus, food presentation in aversively trained animals elicited a discharge in neurons involved in triggering the withdrawal responses in the snail.

4.1.2. Conditioning in semi-intact preparation

In a series of 26 experiments we investigated a possibility to elaborate associative changes in preparation [14]. All preparations were made from hungry snails. An example of one typical experiment is shown in Fig. 15. After testing the responsiveness to food stimuli in withdrawal interneurons (Fig. 15A), the electrodes were withdrawn (in order to prevent damage during contraction elicited by electric shock), and five pairings of the food (juice) application on the lip with electric shock were performed with 20 min interval. After that the electrodes were inserted back, and the responses were tested for 2–3 h. Before pairing in this experiment the juice application never elicited the pneumostome closure.

On the contrary, quite often a pneumostome opening was observed (Fig. 15A), what is normal for hungry snails. After pairing, the juice application elicited a spike response in the command neurons for withdrawal accompanied by the pneumostome closure (Fig. 15B). Similar results were obtained in 12 preparations, while in 14 experiments in which the juice and shock were explicitly unpaired no significant increase in quantity of spike responses to food application in the withdrawal interneurons was noted. It is essential to note that similar and synchronous spike discharges were recorded simultaneously in all command neurons for withdrawal, what indicates their synergic involvement in withdrawal behavior. Results suggest that food-aversion learning results in significant increase of synaptic response of withdrawal interneurons to a particular type of food presentation, thus evoking the spike discharge and corresponding withdrawal.

In another series of experiments we paired juice application with serotonin (5-HT) bath application. After testing the responsiveness to food stimuli in withdrawal interneurons and neurons involved in feeding (Fig. 16A), three random presentations of the food (juice) application on the lip with 5-HT in bath were performed with 20 min interval. Random presentation of stimuli elicited a small increase of response of metacerebral cell (involved in feeding) to juice application 40 min after last stimuli presentation, but still no response in withdrawal command neuron (Fig. 16B). It is essential to note that immediately after three pairings of juice and 5-HT no response in command neurons was present. The response to food appeared about 40 min after the last pairing, and was even greater when tested 70 min after pairing (Fig. 16C and D).
Similar results were obtained in 18 preparations. The results suggest that the serotonin application may be as effective as an electric shock.

4.1.3. Motivational factor and learning

In behavioral experiments in which a conditioned aversion to food was elaborated, it was noted that after learning the hungry (in satiated animals such a reflex cannot be elaborated) animals react to a conditioned type of food as to the noxious stimulus only when it occasionally contacts it. In most cases after food-aversion learning the behavior of a hungry animal resembles the behavior of satiated snails. Using snails with different level of satiety we compared their neural responses to food and noxious chemical stimuli before and after food-aversion training.

Comparison of responses in command neurons for withdrawal to food and quinine applications showed that in sated animals synaptic and spike responses to food are observed in these cells in 80% of cases, while in hungry animals such responses usually can be seen only occasionally (in average in 12% of trials). Quantity of action potentials in responses to quinine increased in sated animals (details in Ref. [83]).Basically, the same level of responsiveness to food stimuli was observed in hungry animals subjected to food aversion training (Section 4.1). It suggests that the synaptic pathways via which the food stimulus elicits a withdrawal responses after food-aversion training already exists in the nervous system, and that pairing of food and shock results in ‘usage’ of existing in the nervous system mechanism switching-on the negative responses to food in sated animals. One of most possible mechanisms of such reversible switching is modulation of effectivity of synaptic inputs, in particular, the synaptic inputs of withdrawal interneurons.

4.2. Serotonin modulation of the aversive behavior and the food-aversion conditioning

In this section the contribution of 5-HT in aversive learning was studied using 5,7-DHT, the ‘neurotoxic’ analogue of serotonin. The associative changes were compared after training in normal and 5,7-DHT-injected snails both on the behavioral level of intact animals and on the cellular level using electrophysiological methods in semi-intact preparations [14].

4.2.1. Effects of 5,7-DHT treatment on feeding behavior and avoidance reaction

Immediately after injection of 5,7-DHT, the locomotion abnormalities were observed: the animals had an increased arousal state, they were crawling intensively, frequently changing their direction. No abnormalities were noted in the behavior of vehicle-injected snails. After several hours, normal behavior reappeared and no visible differences were notable between control and 5,7-DHT-treated animals.

No significant difference was found between the latency of consummatory phase of control and 5,7-DHT-injected animals, whereas a significant difference (M.W., n = 50.80, p < 0.001) was noted in the appetitive phase duration. Following 5,7-DHT treatment, changes were observed in the dynamics of the avoidance reaction (pneumostome closure) in the intact snails, as well as in the number of spikes in the neurons responsible for this withdrawal reaction on the semi-intact preparations. During regular tactile stimulation of the skin the increase in amplitude of pneumostome closure and of the neuronal spike discharge could be observed in control animals but this sensitization of responses was absent in 5,7-DHT-injected animals both at behavioral and cellular level [84].

In separate series of experiments using a double-blind procedure the sensitization of pneumostome closure was tested in intact 5,7-DHT-treated and vehicle-injected animals. In all 5,7-DHT-treated snails this sensitization was absent, but the vehicle-injected animals displayed sensitization, like the control non-injected ones.

The modulatory effect of 5-HT on feeding behavior is well known in gastropods, e.g. as the so called feeding arousal in Aplysia [24]. In the Helix specimens injected with 5,7-DHT in our experiments the increased time duration of the appetitive phase of feeding behavior can be explained as a suppression of this generalized excitatory influence. On the contrary, no change in the consummatory phase was observed, suggestive of another transmitter substance being involved. According to Galanina et al. [35], the 5-HT in H. lucorum cannot trigger the consummatory phase of feeding, whereas dopamine evokes the rhythmic movement of buccal mass, as Wieland and Gelperin [85] demonstrated in other molluscan species.

5-HT might exert its influence not only on central neurons but also directly on muscles too. In our experiments, no abnormality in the locomotion of the animals could be observed visually 2–3 h following the injection. Moreover, the time duration between touch and bite was the same both in control and drug-treated groups, although the locomotion is an essential factor in this period of feeding behavior as well.

4.2.2. Effect of 5,7-DHT treatment on aversive learning of intact animals

The results of behavioral experiments after pairing of food presentation with electric shock (two pairings each day for 8 days) are shown in Fig. 17A. In conditioned animals a highly significant (p < 0.001) decrease of feeding reactions occurred after the learning sessions. The animals refused to touch the food and in some cases a withdrawal reaction was observed after presentation of it. This change of the feeding responses was specific only for the type of food (carrot) paired with the electric shock.

After unpaired presentation of stimuli most of the animals demonstrated no changes in feeding responses. The conditioned 5,7-DHT-injected animals exhibited the same behavioral responses as the unpaired control ones.
suggesting that in these cases no aversive learning was acquired (Fig. 17A). The absence of sensitization of the withdrawal reaction in 5,7-DHT-injected animals in our experiments suggests that in *Helix* (as in *Aplysia*) serotonin takes part in the withdrawal reaction as a transmitter producing facilitation.

In the laboratory of Pavlov [86] it was shown that after training sessions one of the consequences of reinforcement is an unspecific sensitization, and this effect of reinforcement correlates with the number of trials needed for the elaboration of the conditioned reflex. On the cellular level it was confirmed in *Aplysia* by Hawkins et al. [67] that the mechanism of classical conditioning may be elaboration of activity dependent presynaptic facilitation of synaptic transmission between sensory and motorneuron resulting in sensitization of the conditioned reaction. Consequently, if the sensitization process is impaired, no associative changes could be elaborated. Our results in *Helix* are in agreement with this theory: after 5,7-DHT treatment (ablatting the serotoninergic neurons) both the sensitization of the withdrawal reaction and associative aversive conditioning is missing. The feeding behavior of 5,7-DHT-injected, intact animals was visually normal.

To answer the question whether 5,7-DHT treatment could change the performance of consolidated aversive conditioned reaction to food, in a series of experiments eight hungry snails were trained to avoid carrot. After six paired presentations of carrot and electric shock the percent of feeding reactions decreased from 90 to 15%, as in a control conditioned group. Then four randomly chosen snails were injected with 5,7-DHT, and 2 days later a number of feeding reactions to carrot were scored (blind) in all animals. Both control and treated animals responded with feeding in only 10% of reactions. Thus, 5,7-DHT does not impair the aversive behavior per se, but exerts its influence only at the stage of consolidation of aversive conditioning. Another suggestion which can be deduced from presented data is that the 5-HT-containing neurons are not necessary for recall and/or retention of this form of memory.

4.2.3. Two synergic components of memory

The results of 5,7-DHT treatment after elaboration of aversive conditioning suggest that 5-HT is necessary for associative learning (developing a procedural memory) only during the consolidation phase. Retrieval of this type of memory does not require activity of modulatory neurons, and the putative locus of memory is the synapses (including the presynapse) between sensory and command premotor interneurons for withdrawal (Fig. 17B). Quite different results demonstrating that 5-HT is necessary for recall and/or retention were obtained in experiments concerning environmental (contextual) conditioning (see Section 3.5), which can be considered as an independent component of memory. Environmental conditioning (declarative memory) is impaired by suppression of serotonergic system in learned animals, thus suggesting that serotonin-containing cells are involved in retrieval and maintenance of this acquired behavior. Independence of the cellular mechanisms involved suggests that during learning the animal acquires information about the context in which it receives the reinforcement, and independently stores information about certain specific stimuli which are contingent on reinforcement in another locus (Fig. 17B). Adaptive value of independence of these two components of memory is evident: animals can percept the same conditioned stimulus in another context as a novel one, and are prepared in the known context to respond to noxious stimuli. It is essential to note that for both types of memory the locus of plastic changes presumably are the synaptic contacts between sensory neurons and modulatory or command (premotor) neurons (Fig. 17B).

4.3. Positive pairing-specific modulation of synaptic input of withdrawal interneurons

Changes in behavior that correspond to activity of a
single invertebrate nerve cell were described in literature quite early [18,87,88]. Well known examples are lateral giant neurons in crayfish [89], Mauthner cells in fish [90], and neurons controlling withdrawal in mollusks (Section 2.2) [10,91]. These cells were called command neurons and constituted a class of premotor interneurons, whose extracellular activation elicits a goal-directed behavioral response similar to the responses evoked by adequate sensory stimuli [89].

4.3.1. Contingent extracellular activation of the pedal serotonergic cells can serve as reinforcement

Previously published results showed necessity of serotonergic cells for long-term behavioral sensitization and elaboration of context conditioning and food aversion [14, 70,92]. The presence of serotonergic fibers surrounding the soma of withdrawal premotor (command) interneurons with a dense network without synaptic specializations suggesting modulatory influence was clearly demonstrated immunohistochemically [93]. Therefore, we tested the assumption that serotonergic cells can mediate the 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 amplitude of EPSPs in withdrawal interneurons were found when we extracellularly stimulated serotonergic cells located on the border of visceral and right parietal ganglia.

In our experiments the test stimuli to intestinal nerve were applied with 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 EPSPs amplitude decreased usually to the 85–90% level of the initial amplitude with the intertrial interval 20 min [94]. First three test stimuli (pretesting) were followed by paired or explicitly unpaired procedures, 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 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 intertrial interval (10 min apart of test stimuli). The averaged data from 21 experiments (Fig. 18) showed a significant difference ($p < 0.01$, $100$ 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 min after the beginning of 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 during context conditioning [92] and associative learning [14].

4.3.2. One modulatory cell can mediate the reinforcement

The experiments using extracellular stimulation described in Section 3.3.1 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 procedure of training was changed in order to shorten the training session. Test stimuli were delivered with 5 min intervals before and after the pairing session. Increase of test stimulation frequency normally increases the habituation rate [15]. At a frequency 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 [95,96]. A pairing session (Fig. 19A) consisted of five test stimuli with 2 min intervals, and five ‘reinforcing’ intracellular trains to the pedal neuron (Fig. 19B), which were given simultaneously with the test stimuli (paired procedure) or between test stimuli in a pairing session (explicitly unpaired group, Fig. 19A). The EPSP amplitude was not analyzed during the testing session (the gap in Fig. 19C) because the artifacts of tetanization in the paired procedure masked the form of the EPSPs. The ‘reinforcing’ intracellular tetanization consisted of one 10 s duration train of 25–33 ms depolarizing pulses at 15–20 Hz. The current strength (5–10 nA) was suprathreshold. Giant parietal withdrawal (command) interneurons (LPa3 and RPa3) and one of the pedal serotonergic (modulatory) 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. 19B). In most experiments, the training procedure was.
repeated twice: one paired and one unpaired session. In total 27 animals were used for experiments. In 12 of them were used the snails with serotonergic cells previously vitally labeled by 5,7-dihydroxytryptamine [28]. 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 cell Pd4. The difference between responses in paired and unpaired situations was close to zero in 17 snails, and never exceeded the standard error of the mean on averaging. Only when intracellular tetanization of cell Pd4 was used as reinforcement did we observe an increase in EPSP amplitude during the paired procedure (n = 10 snails; Fig. 19C) relative to the experiments with unpaired stimulation of Pd4 cells (n = 6, same snails; Fig. 19C).

Significant difference was observed immediately after the last tetanization (p < 0.05, 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 < 0.01), and up to the 50th minute the difference between paired and explicitly unpaired situations was significant (Fig. 19C). In general, results were similar to those obtained in the experiments with extracellular stimulation of serotonergic neurons (Fig. 18). Thus, intracellular stimulation of only one Pd4 cell can mediate a pairing-specific increase of the amplitudes of the EPSPs in the parietal giant neurons controlling withdrawal behavior [97].

Intracellular staining of the rostral pedal cells with Co2+ (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. 8C), as well as presynaptic cells sending processes in the intestinal nerve [9].

Taking into account that processes of only Pd4 cell overlap with processes and putative synaptic region of the giant parietal (command) cells in neuropile of the parietal ganglia (Fig. 8B and C), it is logical to assume that mainly the Pd4 cell exert 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 when the first withdrawal responses to noxious stimuli can be observed in the embryos (Ierusalimsky, unpublished).

4.3.3. Pedal serotonergic cells constitute a functional neuromodulatory group involved in reinforcement

It is known from earlier studies that the cells located in medio-rostral part of the pedal ganglia, both on dorsal and ventral sides, contain serotonin [27,28,70]. It was shown that distribution of 5-HT-containing cells in the central ganglia of adult Helix using immunochromic method and vital pigment-labeling by 5,7-DiHT (see Section 3.5) coincides. The most important for the present investigation fact is that all pigment-labeled cells show the 5-HT immunoreactivity [31]. Schematically, the location of serotonergic cells on the ventral surface of pedal ganglia obtained by those two methods (our results using the 5,7-DiHT and the literature) are presented in Fig. 4A. In the rostral part of the pedal ganglia were found serotonergic cells only. This area was the only effective for eliciting facilitation of the withdrawal responses using small intensity extracellular stimulation (Section 2.3) [70]. 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.

Each group of serotonergic cells has its target areas where most processes of the cells are branching. With the exception of well-studied giant metacerebral serotonergic
facilitates synaptic responses in the underlying network. Modulatory system for the withdrawal behavior, which their reinforcing role in this behavior. Conditional depolarization of the Pd4 cell elicits pairing-specific increase in the amplitude of synaptic inputs to premotor withdrawal interneurons (Fig. 19), suggesting increase in behavioral response. We suggest here that a single Pd4 cell can affect presynaptic to the withdrawal interneurons sensory neurons, or synapses between sensory neurons and withdrawal interneurons shown to be present in the same neuropile [9].

Participation of individual modulatory cells in modifications of behavior was shown in different invertebrates. Intracellular stimulation of identified cerebral Aplysia neurons CB1 produced facilitation of the EPSPs from siphon sensory neurons to motor neurons suggesting participation of these individual serotonergic cells in mediation of presynaptic facilitation [101]. There are several published examples of identified neuromodulatory interneurons, which serve the 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 axo-dendritic arborizations. This neuron responds to sucrose (reward) with long-lasting excitation, and its depolarization substitutes for the reward in single-trial conditioning [102]. It was clearly shown in cultured Aplysia neurons that temporal pairing of presynaptic activity and serotonin application enhances facilitation at sensory-motor neuron synapses [100,103]. Activation of an identified modulatory cell (slow oscillator) in Lymnaea stagnalis elicited associative enhancement of fictive feeding response [104].

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 [14,70]. Intracellular depolarization of this cell changed the effectiveness of synaptic input in withdrawal interneurons, while the hyperpolarization of Pd4 cell decreased the rate of spontaneous activity in interneurons. Conditional depolarization of the Pd4 cell elicits pairing-specific increase in the amplitude of synaptic inputs to premotor withdrawal interneurons (Fig. 19), suggesting increase in behavioral response. We suggest here that a single Pd4 cell can mediate the aversive reinforcement in snail. This cell can be viewed as a ‘delegate’ neuron from 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.
4.4. Negative pairing-specific modulation of the withdrawal interneurons synaptic input

Opposite to ‘positive’ modulation (increase) of synaptic input of the withdrawal interneurons by the serotonergic cells (Section 3.5), it was shown that extracellular stimulation of mesocerebral cells decreases the amplitude of synaptic input in the same withdrawal (command) interneurons [46]. In this section, we investigated modulatory properties of peptidergic mesocerebral cells exerting ‘negative’ short-term effects on amplitude of synaptic input in withdrawal interneurons. The properties were investigated in the situation when synaptic inputs to the premotor interneurons for withdrawal were paired or explicitly non-paired with activation of modulatory cells.

A neuronal network that consists of various types of peptidergic neurons controls male copulatory behavior of the gastropod snails. Among them is a cluster of neurons in the anterior lobe of the cerebral ganglia (mesocerebrum, MsCer) innervating the penis complex and various central neurons affecting the mating behavior [105]. This functional cluster was shown to contain in Helix several peptides presumably involved in the mating behavior: FMRFamide [44,106], APGWamide and other peptides processed from the APGWamide propeptide [107,108,130] (Lymnaea, Helix), enkephalin-like peptides [106,109]. Extracellular activation of mesocerebral neurons involved in male sexual behavior suppressed for several minutes the amplitude of synaptic responses in the premotor withdrawal interneurons [7].

4.4.1. Contingent extracellular stimulation of mesocerebral cells

In a series of 30 experiments it was observed that stimulation of either nerve paired with the MsCer tetanization significantly ($p < 0.01$ after five pairings) decreased amplitude of synaptic response to the paired test input relative to the unpaired one (Fig. 20A and B). Significant difference between the amplitude of responses in inputs contingent with extracellular tetanization of MsCer and non-contingent inputs lasted for 2–3 h (Fig. 20). In order to test whether the inputs (nerves) used for the test stimulation may behave differently in situation with contingent extracellular tetanization, we alternated the nerve that was used for contingent stimulation in different experiments. The results obtained showed independence of effect—pairing-specific long-term decrease of synaptic responses to the test stimulation—of the type of test input (Fig. 20A and B). An example of pairing-specific changes in the complex EPSPs amplitude elicited by unpaired (left) and paired test stimuli is shown in Fig. 20C.

It is well known that cells in MsCer contain several peptides, and between them the enkephalin-like immuno-reactive neurons were found [106,109]. We tested in a series of 14 experiments whether a contingent bath application of a synthetic met-enkephalin will produce significant difference in responses to contingently and non-contingently stimulated inputs. Met-enkephalin was added in final concentration $10^{-6}$ M to the saline 1 s prior the test stimulation (1 s is estimated time for reaching the CNS in our chamber), and was washed out in the middle of the 10 min interval between consecutive test stimuli. It was found in 14 preparations that the bath application of met-enkephalin can serve as a contingent stimulus and also elicits a significant ($p < 0.05$) pairing-specific changes in complex EPSPs amplitude (Fig. 21) quite similar to the changes evoked by contingent MsCer tetanization (Fig. 20).
contingent application of met-enkephalin stimulated with synaptic input to the withdrawal intermediate reinforcement in situations when it is contingently present. In paper we showed that the MsCer stimulation can withdraw responses during mating between snails. In the intact preparations, extracellular stimulation of mesocerebral cells elicited a decrease in the number of action potentials evoked in parietal giant interneurons and a corresponding decrease in the amplitude of pneumostome potentials evoked in giant parietal interneurons and a cerebral cells eliciting a decrease in the number of action potentials normally triggered by tactile contacts. In semi-intact preparations, extracellular stimulation of mesocerebral cells elicited a decrease in the number of action potentials evoked in giant parietal interneurons and a corresponding decrease in the amplitude of pneumostome closure (part of withdrawal behavior) [46]. Described effects provide a partial explanation for the suppression of withdrawal behavior [105]. Electrical stimulation of the right MsCer with an extracellular suction electrode caused contraction of the penis sheath and the 'love dart' sac. Intracellular stimulation of individual mesocerebral neurons caused contractions of reproductive organs. These effects are mediated by axons that travel directly from the MsCer to the suboesophageal ganglia [105]. During mating, snails exhibit a marked suppression of the defensive withdrawal responses normally triggered by tactile contacts. In intact preparations, extracellular stimulation of mesocerebral cells elicited a decrease in the number of action potentials evoked in giant parietal interneurons and a corresponding decrease in the amplitude of pneumostome closure (part of withdrawal behavior) [46]. Described effects provide a partial explanation for the suppression of withdrawal responses during mating between snails. In the present paper we showed that the MsCer stimulation can mediate reinforcement in situation when it is contingently stimulated with synaptic input to the withdrawal interneurons (Fig. 20). Contingent application of met-enkephalin (10^-6 M, some mesocerebral cells are enkephaline-like immunoreactive), shown to decrease the amplitude of synaptic responses, also selectively decreased synaptic responses to conditioned nerve stimulation in the premotor interneurons for hours (Fig. 21).

Summarizing the obtained results, it is essential to note that in all experiments the mesocerebral stimulation caused a decrease of amplitude of synaptic input to the premotor interneurons, while stimulation of pedal serotonergic cells led to opposite effect: increase of amplitude of synaptic input to the same withdrawal interneurons. Thus, two modulatory inputs exert pairing-specific effects that influence the same synaptic connection in opposite directions, what may underlie the long-term up- and down-regulation of behavioral responses.

5. Applicability of the reinforcement concept to studies in simple nervous systems

The reinforcement concept is widely used in studies of learning and memory mechanisms which have been performed in the last few decades, not only at the behavioral level, but also in model systems, such as brain slices, invertebrate isolated nervous systems and synthetically connected neurons in vitro and in vivo. In these studies the terminology elaborated for animal behavior has mainly been used. In this section analyzing the limitations and applicability of the behavioral concept of reinforcement to neurophysiological experiments in model systems is aimed.

5.1. Reinforcement in learning theories

Analysis of the abundant literature concerning theories of learning in psychological terms (i.e. describing the behavioral events), allows us to find common ideas in each approach, and to relate them to the contemporary concept of reinforcement.

Historically, the term reinforcement has had many differing meanings. Spencer’s theory of learning [110] was the first systematic attempt to offer explanations for the differential strengthening of behavioral patterns. His theory implied that an organism would obviously tend to repeat actions that brought pleasure, and desist from those which brought pain. In cases when pleasure accompanied actions that were beneficial for survival, or pain accompanied injurious actions, the animal had an advantageous position for natural selection. Spencer’s theory was the first to imply the existence of a reinforcement process which was necessary for differential change in behavior.

The term ‘reinforcement’ was first introduced into the psychophysiological arena in the laboratory of Pavlov, and appeared in the world literature after publication in 1927 of a translation of his “Twenty Years of Experience” first published in Russian in 1923 [111]. Reinforcement in this theory of learning is, by implication, a property of the unconditioned stimulus which exerts the ‘reinforcing action’. It was noted in the paper devoted to the problems of reinforcement by Pavlov’s student [112] that in Pavlov’s publications there is no special work analyzing reinforcement as a concept, which suggests that Pavlov never regarded this problem as an independent one.

In the ‘Law of Effect’ formulated by Thorndike [113] it is clearly stated that the nervous system is so constructed as to lead to the strengthening of those connections which have been active just prior to a satisfying event, and to the weakening of those connections which have been active prior to annoying events. This change of effectiveness of stimulus–response (S–R) connections implies the existence...
of some sort of nervous process influencing the S–R connections, upon which the behavioral effect of contingent stimuli depends.

In the studies by Hull [114], which are to some extent a continuation of Thordike’s ‘Law of Effect’, the concept of a biological need and its associated drive were introduced. Drive reduction, increasing the probability of S–R association, has the properties of reinforcement in this theory. Skinner [115] introduced a concept of events which may serve as a ‘reinforcers’, and which are not connected exclusively with stimulus or reaction. A concept of ‘emitted operants’, which can be brought under the control of a stimulus by arranging for the emission and reinforcement of the operant in the presence of the stimulus, completed his description of behavior.

It is essential to note that, in all the theories of learning just mentioned, there are common properties: Stimulus, Response, and Motivation are mentioned in all of them. The context may differ somewhat, but the involvement in learning of these phenomena is acknowledged. Based on their own experimental experience, different authors assign the reinforcing properties either to Stimulus or Reaction or Motivation, but in all cases the argument is strong and valid. It suggests two possibilities: Only one of the authors is right, which is improbable, or all of them are right, which means that reinforcement cannot be attributed to only one of the phenomena mentioned.

Our next question is: “What is reinforcement? Is it an independent behaviorally described phenomenon, or is it a state of the organism which can be elicited in different ways?”

5.2. Reinforcement: an independent phenomenon or a state of the organism?

As has been noted, in publications of Pavlov, reinforcement was never considered as an independent phenomenon [111,112] in spite of the fact that its importance was mentioned in each paper. A prominent student of Pavlov’s, Anokhin, also stressed the role of reinforcement [116], but never treated it independently in his papers. A brilliant analysis of reinforcement as a concept was made in a chapter of a book ‘Reinforcement and Behavior’ by Walker [117]. He compared the influence of reinforcement on studies of learning with ‘The One Ring’ from Tolkien’s ‘The Lord of the Rings’. The possessor of the One Ring could exercise mastery over every living creature, but the use of the ring inevitably corrupted the person who used it. In fact, using reinforcement as a concept has some advantages in the interpretation of behavior, but using it undermines the conceptual framework. Walker suggested destruction of this powerful instrument in order not to distort the learning mechanisms with an excessively mechanistic interpretation of its functioning. Indeed, reinforcement is a mechanistic concept used to explain why repeated associations are strengthened. The simplistic way to explain the strengthening is to suggest the existence of some sort of a selective ‘glue’ which is present in the organism. But all searches for this glue as a physical entity in the organism were in vain, as well as the search for the physiological basis of reinforcement which appeared to be too diversified.

From our point of view, it is necessary to define the term reinforcement operationally. Analysis of the literature suggests that either external stimuli, behavioral response, or motivational state of the organism may have ‘reinforcing’ properties. In order to have an optimal description of all known data concerning reinforcement, it is logical to consider a special state of the organism preceding changes in behavior as the ‘reinforcing state’, that can be elicited by presentation of stimuli, behavioral responses or changes in motivation, as has been shown experimentally.

5.3. Simple networks and reinforcement

The aim of the present analysis is to find out whether it is correct and useful to use this behavioral term in investigations of learning mechanisms in model situations when only a small part of nervous system is under the experimenter’s control. Even in the relatively simple nervous system of mollusks, tens of thousands of neurons participate in control of behavior, and, without a conceptual framework of the organization of the behavioral act, it is impossible to approach mechanisms of learning. One of the most complete descriptions of organization of a behavioral act in the traditions of Pavlov’s school was presented in the concept of ‘Conceptual Reflex Arc’ by Sokolov [118]. This concept provides a physiological basis for stimulus perception, integration of information and decision making, realization of motor programs of behavior, and, in addition, a neural base for motivational influences is described. In our work in gastropod snails we used this conceptual framework to describe the avoidance reaction and feeding response (Fig. 22). It is well known that, after pairing of food presentation with noxious stimuli, the snail reacts selectively by avoidance of the type of food associated in time with noxious stimuli [14]. In this short description of the paradigm, we have not used the term reinforcement. More than that, it is not necessary to use this concept, and we cannot find a place for it in the scheme (Fig. 22). But when we want to describe the results of the learning procedure we cannot miss the ‘change in synaptic effectivity’ which is in some sense absolutely equal to Thordike’s “strengthening of connections between stimulus and reaction”. It is essential to note that we can judge whether there was reinforcement or not only after the learning procedure, during testing of the resulting behavior. In the case of aversive food conditioning, the animal ceases to respond with feeding behavior to the type of food associated with noxious stimuli, which indicates that the reinforcement state was invoked.

What is the place of reinforcement in this simple
learning, thus exerting a reinforcing effect. It is for the unconditioned stimulus in associative olfactory intracellular stimulation of an identified neuron substituted can investigate this network and try to find out which part of to some particular block on the scheme. Nevertheless, we net schematically drawn in Fig. 22, but we cannot assign it reinforcement', as is often found in literature. It suggests reinforcement only post factum, and we cannot 'apply the state was invoked. Therefore, we conclude that there was a difference between two possibilities. The first possibility refers to the case when testing shows absence of changes in behavior. The conclusion is drawn that the reinforcement state was not achieved in the learning procedure. The second possibility is the case in which the learning procedure changed the behavior, which means that the reinforcing state was invoked. Therefore, we conclude that there was a reinforcement only post factum, and we cannot 'apply the reinforcement', as is often found in literature. It suggests that the reinforcement is a change in a state of the nervous net schematically drawn in Fig. 22, but we cannot assign it to some particular block on the scheme. Nevertheless, we can investigate this network and try to find out which part of the nervous system creates this reinforcement state.

5.4. Cellular origin of reinforcement in a simple network during learning

There is only one case described in the literature in which intracellular stimulation of an identified neuron substituted for the unconditioned stimulus in associative olfactory learning [119], thus exerting a reinforcing effect. It is essential for our analysis to note that this identified neuron (in a honeybee) contained octopamine, and was shown to be involved in modulation of behavior (sensitization) in this animal [102]. The neuromodulatory substance serotonin was shown in *Aplysia* to participate in associative conditioning [120].

In *Helix*, the modulatory neurons are also the main candidates for exerting the reinforcing effect. In this section we will try to discuss data concerning cellular locus in the network whose activity may correlate with the state of reinforcement, using the example of aversive conditioning in the snail described in detail in Section 4 [14,15,97]. Only the main behavioral component of aversive learning will be considered, namely the aversive response to food paired previously with noxious stimuli. As a first step of analysis one should determine the functional group of cells, whose activity changes in correlation with existence of reinforcement. It is well known that modulatory neurons address their influence to a number of functionally different neurons, therefore the changes of their activity would synergically influence several behaviors.

As a result of learning according to this scheme, the change should take place only in certain sensory inputs of a command cell. Where can the reinforcement be 'applied' in this case? It was established in electrophysiological experiments that, due to the learning procedure, a novel response appeared in command neurons for withdrawal, and a corresponding behavioral withdrawal to food presentation appeared (Section 4.1) [14]. A morphological connection between sensory neurons reacting to food presentation and command neurons existed before the learning procedure, but was weak and evoked only the subthreshold responses. The factor, which can influence the synaptic input of command neurons, may have a relation to reinforcement. The best candidates for strengthening the connection between sensory input and spike discharge in command neurons are serotonergic modulatory neurons, which have been well described in the snail (Section 2.3) [70]. During elaboration of the conditioned reflex, the coincidence of conditioned sensory input and modulatory influence may serve as a condition for selective changes in sensory input of the command neurons. In terrestrial snails it was shown that activity of these modulatory cells can be affected by strong external stimuli, and motor performance also depends on their activity [70]. Therefore, in the particular example of aversive learning of the snail, the most likely group of cells—the activation of which may correlate to the existence of reinforcement—is a functional class of serotonergic modulatory cells. These modulatory cells were shown to react to such contextual cues as acidity of the substrate, and the level of glucose corresponding to satiation level (Zakharov, unpublished). Modulatory cells directly participate in elaboration and reproduction of contextual conditioning which is an implicit part of conditioning (Section 3.5) [92]. In full correspondence to this conclusion are the results showing that extracellular stimulation of the modulatory cells can serve as a reinforcement (Section 4.3), and even intracellular stimulation of one cell can elicit pairing-specific changes in effectivity of synaptic inputs of command neurons for withdrawal (Section 4.3). In a more general sense, these cells may be called motivational. Therefore, the most likely candidates for neurons creating the state of reinforcement are the neuromodulatory cells related to motivation.
5.5. Speculation: reinforcement = emotions

Neuropsychologists widely accept that the appearance of an emotional state of the organism improves learning and memory. Let us assume that the reinforcement state is the same as the emotional state. In this case the main problem for invertebrates will be the question: Is it possible for the snail to consider a certain situation as emotionally positive or negative? In other words, can the snail feel pleasure? It is evident, that the human experimenter never can place itself in the snail’s brain, but in neurobiology an experimental technique—self-stimulation—can provide an answer regarding whether the situation is pleasant for the animal, or causes a distress.

In the phenomenon of self-stimulation, an animal receives direct electrical stimulation of the brain as a consequence of its operating a manipulandum. If the electrode is implanted in certain areas of the brain, the animal repeatedly self-stimulates [121,122]. Since its discovery [121], numerous experiments have confirmed the phenomenon of self-stimulation in various vertebrate species [123]. However, self-stimulation itself, as well as its relation with learning and reward, remains to be explained by mechanisms at the cellular level [124]. To develop a new approach to this problem, we investigated whether a snail, with its relatively simple and technically advantageous nervous system, will self-stimulate. Freely moving animals with chronically implanted electrodes were used, and the rewarding properties of a contingent extracellular stimulation of a certain cellular groups in semi-intact preparations were investigated [7,125].

To receive stimulation, the snail was required to displace the end of a rod (pedal), thus closing a switch. Usually, the snail would first sense the rod with its tentacles, then raise its head to explore the rod with its lips and mouth, displacing the rod during exploration. Comparison of changes during 40 min without reinforcement, and in two different groups of snails (with electrodes in mesocerebrum and in parietal ganglia), shows a significant effect of the self-reinforcement on the on-going behavior.

The opposite direction of changes in the response rate in snails with electrodes in the parietal ganglion vs. those with electrodes in the mesocerebrum provides additional evidence for the difference in behavioral effects of snail brain stimulation in two different zones. Investigations of results of stimulation of certain areas of the snail brain associated with spontaneous movements demonstrated that spontaneous movements can be modulated either way depending on what is reinforced: the movement or its absence [125].

Neural substrates mediating reward (or reinforcement), have been identified in vertebrate brains using the self-stimulation procedure. The medial forebrain bundle at the level of the hypothalamus is the most effective site [126]. However, no complete neural circuits have been delineated, nor has it been possible to identify any individual neurons whose participation is essential. These difficulties have so far prevented any mechanistic explanation of self-stimulation at the cellular level. The identification of the mesocerebrum as a site of reward in snails offers the possibility of cellular studies, because the neurons in this region are large (diameters up to 80 mm) and easily accessible for intracellular investigation. Interrelations between mesocerebral cells and parietal giants were investigated [46], and it was shown that stimulation of the mesocerebrum causes suppression of spiking in the parietal command neurons in response to tactile stimulation of the skin. In addition to the effects observed at the level of command neurons, a second, independent control over withdrawal is brought about by the inhibition of neurons that are capable of sensitizing the afferent excitation of the same parietal command neurons [46]. Unfortunately, these data cannot be linked directly to emotionally positive effects exerted by the same mesocerebral cells, and this impossibility is caused by the absence of a cellular hypothesis for emotional processes, with only a small overlap of behavioral physiology and cellular physiology. We hope that further investigation of emotionally dependent behaviors in animals with relatively simple behaviors and accessible for cellular analysis nervous system would increase this overlap.

The experiments reported here suggest the possibility that snails learn the rewarding properties of electrical stimulation during the course of a session, or a series of sessions. It means that the interpretation of reinforcement as equal to emotion is valid for the invertebrate animal as well.

6. Conclusions and perspectives: induction of learning and retention of changes, hardwired synaptic connections and modulation

Going by the reductionist way and studying more and more local events in the network, cell, synapse, membrane, ionic channel, we inevitably are losing the functioning of the brain as a whole entity, which may have emergent properties not attributable to its parts. Having in mind to locate the sites of plasticity, it is interesting to compare the behavioral learning and the local synaptic plasticity in order to explicitly delineate the limits of discussed phenomena.

During elaboration of the food-aversion conditioning we can observe a number of changes in behavior. Before training, in response to food was activated a complex feeding behavior, but no withdrawal responses. After training, the same stimulus may elicit two types of behavior. First type of behavior was called ‘conditioned fear’, and is expressed in increase in amplitude, decrease of threshold of withdrawal responses. The second type of behavior, which is seen when the food is in contact with the chemoreceptors (in our experiments in terrestrial snails we often used liquid food application), is characterized by appearance of a complex withdrawal response and lack of feeding reaction. After development of a food-aversion behavior to a particular type of food, the information about presence of food still reaches the
neurons involved in feeding, but it is changed, because feeding response is not activated. It means that due to conditioning some unidentified neural group is activated by presentation of reinforced type of food, which inhibits the feeding network. One may presume that the same neurons inhibit the feeding behavior during strong noxious stimulus in naive animals. In our analysis of learning we in fact discussed only the mechanism of modification of synaptic input of one class of neurons (command neurons for withdrawal) in different conditions. We do not consider it possible to claim that the observed changes are responsible for the behavioral learning. Without any doubt the mechanism of learning cannot be localized in one type of synapses, because behavioral change requires synergic activity in all the brain. That is why in this work we use mostly the term plasticity, as more generic and related to the investigated phenomena. On the other hand, it may be quite possible that changes in one type of synaptic contacts may cause the events which are expressed on behavioral level as the learning.

Still the implicit mechanistic (which is the simplest) way of thinking requires an answer to a question: are there loci where memory is?

In this section, based on the discussed data, once more we will try to locate the neurons or connections, changes in which may be responsible for only one component of food-aversion learning: withdrawal reaction to food (procedural memory). But now we will try to approach this problem from the different side.

Let us imagine, that in the network exists a pool of special ‘Learning Neurons’, which after pairing of two stimuli begin to react on a suprathreshold level to conditioned stimuli. What kind of properties these cells should have? Let us try to take as a standpoint the phenomenology of the food-aversion conditioning. If we will consider only one effector (tentacle contractor muscle), then after learning the same response (tentacle contraction) as was seen before to a specific range of stimuli, will be seen in response to a conditioned one. In fact, the only change is an increase of a set of stimuli which evokes the tentacle contraction by the new one, and there in no limits for addition of another stimuli to this set except abilities of receptors to distinguish parameters of sensory stimuli. Even from this simple analysis it is evident, that the putative ‘Learning Neuron’ should have the potential capability to obtain information of all modalities that potentially can evoke the unconditioned response after learning. Just at this stage it becomes evident that primary sensory neurons or sensory neurons that receive information of only one modality cannot be the candidates for this role. Motoneurons also are not a good candidates, because most part of them are involved in different networks, participate in several movements, and the long-term changes in them would be reflected in overall behavior, what do not happens. In other words, the ‘Learning Neurons’ should be related at their output only to a particular unconditioned movement, should receive polymodal convergent input, and be able to trigger certain behaviors. It is well known that modulatory neurons address their influence to a number of functionally different neurons, and also are not likely candidates. The most conforming candidates are the command neurons (Fig. 22), whose function is connected only with one particular functionally significant movement to a certain set of external stimuli. After the conditioned reflex elaboration, the command neuron responds with a spike discharge to the previously subthreshold stimulus that was reinforced. Note that neither perceptive, nor performing parts of the nervous system are not influenced in this case.

As a result of learning according to this scheme, the change should take place only in certain sensory (synaptic) inputs of the command cells. During development (induction) of memory, the condition for selective change may be a coincidence of conditioned sensory input and modulatory influence from the state system (see Section 2). As it was shown in Sections 4.3 and 4.4, in terrestrial snails the serotonergic neurons positively modulate the synaptic input of withdrawal interneurons, and their activity is a necessary condition for pairing-specific changes, while modulation by mesocerebral cells of the same synaptic input is also pairing-specific, but negative.

Therefore, a rather simple conclusion that follows can be made on the basis of discussed results. Modulatory systems and their ‘soft’ influence are not necessary for eliciting a reflex response to external stimuli. Synaptic connections of neurons represent a ‘hard-wired network’ underlying the responses to external stimuli. Changing these hardwired connections requires participation of modulatory systems, and, what is most important, modulatory influences have a reinforcing properties. This is proved by selective impairment of the modulatory (serotonergic) system that impaired the ability to acquire food-aversion tasks, and by pairing-specific effects of the activation of the modulatory systems. Thus, two principles of the network organization (synaptic connection and mostly non-synaptic modulation) complement each other in the nervous system.

It may be concluded that retention of memory requires changes in synaptic connectivity, while induction is mostly due to contingent activation by external stimuli of the modulatory systems.

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