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The activity of isolated snail neurons controlling locomotion is affected by glucose

Varvara Dyakonova[1], László Hernádi[2], Etsuro Ito[3], Taisia Dyakonova[1], Igor Zakharov[1] and Dmitri Sakharov[1]

[1]*Laboratory of Comparative Physiology, Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia*
[2]*Balaton Limnological Institute, MTA Centre for Ecological Research, Tihany, Hungary*
[3]*Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, Shido, Sanuki 769-2193, Japan*

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The involvement of serotonin in mediating hunger-related changes in behavioral state has been described in many invertebrates. However, the mechanisms by which hunger signals to serotonergic cells remain unknown. We tested the hypothesis that serotonergic neurons can directly sense the concentration of glucose, a metabolic indicator of nutritional state. In the snail *Lymnaea stagnalis*, we demonstrate that completely isolated pedal serotonergic neurons that control locomotion changed their biophysical characteristics in response to glucose application by lowering membrane potential and decreasing the firing rate. Additionally, the excitatory response of the isolated serotonergic neurons to the neuroactive microenvironment of the pedal ganglia was significantly lowered by glucose application. Because hunger has been reported to increase the activity of select neurons and their responses to the pedal ganglia microenvironment, these responses to glucose are in accordance with the hypothesis that direct glucose signaling is involved in the mediation of the hunger-related behavioral state.

Key words: behavioral state, serotonergic neuron, extrasynaptic release, volume transmission, *Lymnaea stagnalis*

Biophysical properties of individual neurons are known to be regulated by intra- and extracellular mechanisms, and depend upon the behavioral state of the organism [1–3]. Recently, we demonstrated that the hunger behavioral state could produce long-term changes in individual serotonergic neurons of the pond snail *Lymnaea stagnalis* that persist even after neurons are isolated from the nervous system [4–6]. Pedal serotonergic neurons of the pedal A (PeA) cluster that control locomotion [7] showed higher activity and depolarized membrane potentials after being isolated from the nervous systems of food-deprived animals. We also reported that the chemical neuroactive microenvironment of the pedal ganglia changes in accordance with the nutritional state of the animal, and produces predictable changes in single isolated neurons placed in its vicinity [3,8]. PeA cells isolated from fed preparations displayed higher excitation when placed near the PeA cluster of food-deprived snails. These hunger–induced effects can be explained by the elevated extrasynaptic serotonin release produced by an increased synthesis of serotonin in pedal serotonergic neurons [3,8–13]. The role of serotonin in mediating a hunger/feeding behavioral state has been suggested and described in many invertebrates [14–18], including mollusks [3,5,19,20]. However, the mechanisms by which hunger signals to serotonergic cells remain unknown.

Here, we tested the hypothesis that serotonergic neurons can directly sense the concentration of glucose, a metabolic indicator. Glucose is the preferred carbon and energy source for most eukaryotic cells [21–23]. In *Lymnaea*, it has been demonstrated that the hemolymph glucose concentration may change dramatically in response to hunger and satiety, from 57 μg/ml in starved specimens to 760 μg/ml in satiated ones [24], reflecting the quality and quantity of the food

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Abbreviation: 5-HTP, 5-hydroxy-L-tryptophan

Corresponding Author: Varvara Dyakonova, RAS Institute of Developmental Biology, Vavilov Str. 26, 119991 Moscow, Russia.
e-mail: dyakonova.varvara@gmail.com

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consumed. The concentrations showed variations in accord with seasonal temperature [25]. Glucose depolarized neurosecretory cells produce an insulin-like hormone, suggesting that the hormone-producing cells are endowed with glucose receptors [26].

Materials and Methods

Animals

Mature specimens of *Lymnaea stagnalis* (2–3 months old, 2.5 cm length) were taken from a breeding colony kept in dechlorinated tap water at room temperature and fed on lettuce. Central ganglia were dissected from snails anesthetized with an injection of 0.1 mM $MgCl_2$. The central ganglia were placed into a 2.5 mg/ml of pronase E (Sigma) in snail physiological solution (50 mM NaCl, 1.6 mM KCl, 4 mM $CaCl_2$, 8 mM $MgCl_2$, 10 mM Tris, pH 7.6) for 15 min, washed in the physiological solution and pinned to Sylgard in a 4 ml chamber. The connective tissue sheath was then removed from the pedal ganglia.

Intracellular recording

Visual identification of the PeA neurons (30–90 µm, PeA2, PeA4, PeA8, PeA11 according to classification [7]) was performed based on their location, size and coloration (Fig. 1A). The neuron was impaled with a standard glass microelectrode (10–20 MΩ filled with 3 M KCl). A standard setup for microelectrode recording was used. The electrophysiological recordings were stored in computer files using a homemade program [5,6,8,13].

For neuron isolation, we utilized previously developed methods [13,27]. Using the intracellular microelectrode as a pull, the neuron was gently pulled out of the tissue until the separation of the proximal neurite from the neuropile was achieved. The electrical activity of the cell was monitored during isolation. Cells that demonstrated membrane injury were not used in the experiments. The isolated neuron (n=12) was placed into a continuous stream of physiological solution (0.75 ml/min). Glucose at a concentration of 250 µg/ml was added into the stream. The firing rate of a neuron was recorded prior to and after 5 min of glucose application.

In eight experiments, the effect of glucose pretreatment for 7 minutes on the isolated neuron's response to the nearby PeA cluster was tested. Our approach was developed based on previously described methods for the detection of extrasynaptic release of neuroactive compounds from the ganglia of *Lymnaea* [8,28]. Two CNS preparations were pinned down in one chamber. The first preparation was used as a source of biosensors (isolated PeA cells), the second for the investigation of the neuroactive microenvironment of the pedal ganglia. The PeA neuron impaled with the microelectrode was isolated from the first CNS preparation and placed at a distance of at least one ganglion-diameter from the CNS for 2 minutes. Then, it was moved to the intact pedal A cluster of the second CNS preparation at a distance less than half-cell size for 2 minutes and then replaced. This procedure was repeated several times in one experiment. In each position, the electrical activity of the biosensor was measured. In each experiment, 2–3 biosensors were used to check the concordance of biosensor responses. Experiments were performed in a continuous stream of either snail physiological solution (control) or 250 µg/ml glucose in physiological solution.

Statistics

The significance of differences in spike frequency was tested by the paired Wilcoxon signed-rank test for dependent samples using the STATISTICA program (StatSoft Inc. 1993, release 4.3). All values are given as the mean with the standard error and level of significance.

Results and Discussion

To test the hypothesis that metabolic signals present in a changing nutritional state may directly affect the serotonergic cellular response, we tested the effect of glucose on the activity of PeA neurons. Glucose concentrations can vary in accord with feeding state over a very broad interval (57–760 µg/ml) in *Lymnaea* [24]. The responses of isolated PeA neurons (n=12) exposed to an application of glucose at 250 µg/ml in a snail saline stream were tested. This concentration increases the osmolarity of the solution by approximately 2%.

Glucose caused the slow hyperpolarization of isolated PeA neurons with the maximal effect being observed at 3–5 minutes of application (Fig. 1B, C). A paired Wilcoxon test indicated a significant difference in the firing rate prior to and after 5 min of glucose application (n=12, z=3, $p<0.01$).

However, the same glucose concentration failed to produce a statistically significant effect on "nonisolated" PeA cells in the CNS preparation (n=10, Fig. 1B, C). This might be explained by lower glucose receptor availability *in situ* because the cell membrane is not entirely exposed in these conditions or by the effects of other glucose-sensitive neurons in the CNS on the PeA cells. Another explanation is that insulin is not present in isolated neurons, which aids the uptake of glucose and, therefore, allows glucose to exert more profound effects on the isolated cells than on those *in situ*.

In spite of the absence of a statistically significant effect of glucose on the activity of PeA neurons in the CNS, the pretreatment of the pedal ganglia for 7 minutes with glucose resulted in a significantly weaker response measured by a biosensor near the PeA cluster (n=8, Fig. 2 A, B). The absolute mean frequency of electrical activity of isolated neurons near the PeA cluster was significantly lower in preparations pretreated with glucose (Fig. 2B and C, $p<0.05$, Wilcoxon signed-rank test). The amplitude of the isolated neuron response to the PeA cluster approach was also lower in glucose-treated preparations (Fig. 2B and C, 9±2 spike/min versus 19±4 spike/min in control, $p<0.05$, Wilcoxon signed-

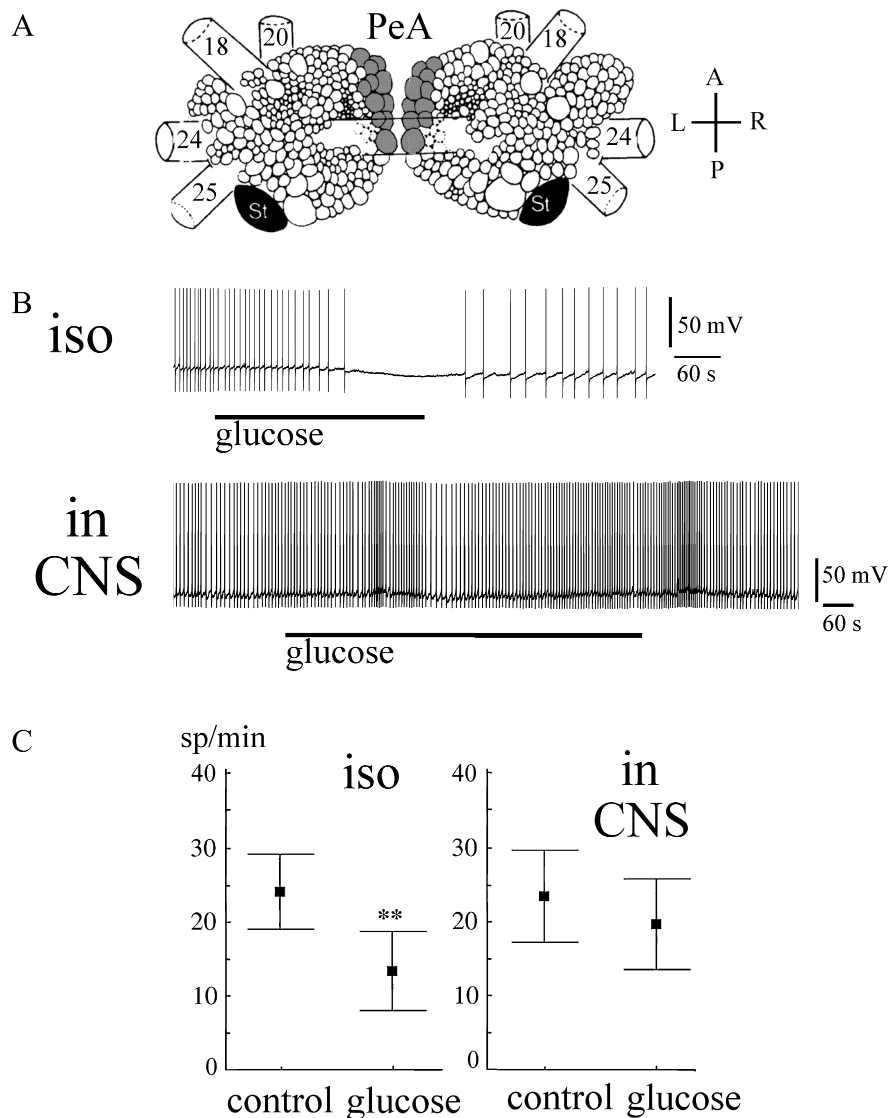

Figure 1 Effects of glucose on the activity of isolated and non-isolated serotonergic neurons of the PeA cluster. A. The positions of the PeA cluster neurons (shaded) at the dorsal surface of the paired pedal ganglia of *Lymnaea stagnalis*. 18, superior pedal nerve; 20, medial pedal nerve; 24, cerebro-pedal connective; 25, pleuro-pedal connective; St, statocyst St, statocyst; A, anterior; P, posterior; L, left; R, right; Modified from Slade et al., 1981. B. The records of activity of isolated (iso) and non-isolated (in CNS) PeA neurons prior to and after bath application of glucose. C. The activity of isolated (iso) and non-isolated (in CNS) serotonergic PeA neurons [mean with standard error prior to (control) and after glucose treatment (glucose), ** $p<0.01$, paired Wilcoxon test].

rank test). This finding suggests that glucose influences volume chemical signalization from the pedal ganglia by additional mechanisms beyond its effect on electrical activity. The present observation that extrasynaptic release of neuroactive compounds including serotonin increases the firing activity of isolated PeA neurons even after glucose pretreatment suggests that serotonin can partially compensate for the hyperpolarizing effects of glucose in the CNS.

Extrasynaptic neurotransmitter release plays an important role in interneuronal communication in the mammalian brain [29] and in invertebrates of various taxa [30–33]. Nutritional state-dependent extrasynaptic release of serotonin and other substances from the pedal ganglia of *Lymnaea stagnalis* has been suggested to play a role in the cooperation of PeA cells and in neurohormonal communication [6,8,13]. The effect observed here of glucose on intensity of extrasynaptic release from isolated ganglia is a unique example of a link between a concentration of a metabolic indicator of a nutritional state and the neuroactive chemical microenvironment of the ganglia.

In molluscs, satiation-induced inhibition of feeding is believed to be mediated by the mechanical stimuli that result from filling the gut with food [34]. In *Lymnaea*, gut dilation activates the mechanosensory cells, which inhibit modulatory and pattern-generating neurons and also activate radular

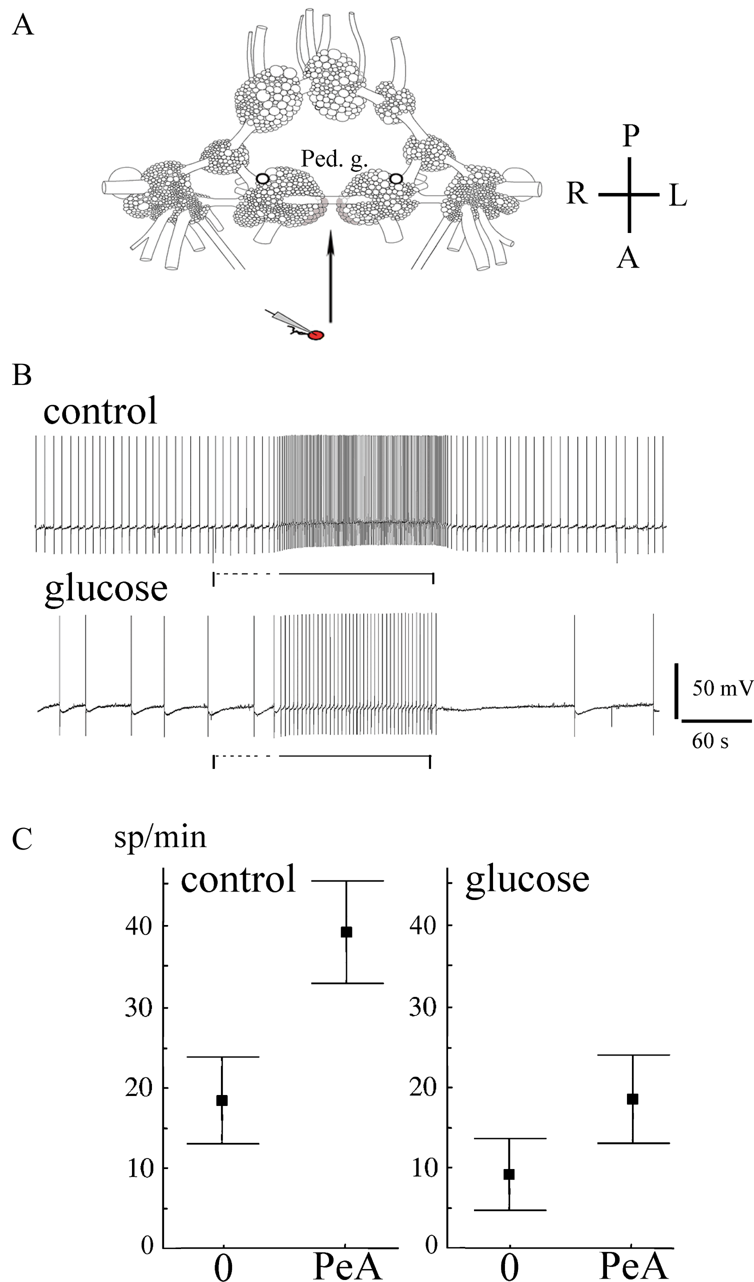

Figure 2 Effects of glucose on the activity of isolated PeA neurons and their responses to the nearby PeA cluster of pedal ganglia. A. A schematic representation of the experimental procedure: the isolated neuron impaled with the microelectrode was placed at a distance from the pedal ganglia of an isolated CNS then moved to the pedal A cluster (shaded) at a distance less than half-cell size for 2 minutes and replaced. A, anterior; P, posterior; L, left; R, right. B. The response of isolated serotonergic PeA neurons to the nearby pedal A cluster prior to (upper record) and 7 minutes after (lower record) bath application of glucose. The vertical lines mark the start of biosensor movement, the horizontal dashed lines mark the movement of the biosensor to the PeA cluster, and the horizontal black lines mark the unmovable state of the biosensor near the PeA cluster. C. The activity of isolated serotonergic PeA neurons (mean with standard error), left to right: prior to glucose treatment away from the pedal ganglia (0) and near the PeA cluster (PeA), 7 minutes after glucose bath application away from the pedal ganglia (0) and near the PeA cluster (PeA). Other statistical data are in the text.

retractor motoneurons [35]. In *Aplysia californica*, the feeding behavior was found to be unaffected by glucose [36], and therefore the mechanical stimulation of the gut remained the only known mechanism of satiety. It was unknown how neurons beyond the feeding system could sense changes in the nutritional state. Later, in some *Lymnaea* neurons from the buccal feeding network, glucose was reported to produce a hyperpolarization *in situ*, while in other feeding neurons there was no effect [4]. The firing activity of the serotonergic metacerebral giant cell (MGC) modulating the feeding sys-

tem was decreased by glucose in the land snail *Helix pomatia*, and this effect could be compensated by extracellular serotonin (Hernádi *et al*., unpublished data). In a recent study in *Lymnaea*, ambiguous data on the direct effects of glucose on food-aversive learning have been obtained [37]. Injection of glucose into food-deprived snails did not affect their learning. However, if these snails were fed on sucrose, they showed learning and memory formation. It was concluded that hemolymph glucose concentration is an important factor in motivating acquisition of food-aversive learning and memory in *Lymnaea*. However, not only glucose but insulin and feeding motor activation are also required for memory formation [37].

Conclusion

The present experiments show that completely isolated PeA neurons decreased their firing rate in response to glucose application. Additionally, the excitatory response of isolated PeA neurons to extrasynaptic ganglia release was also significantly lowered by glucose application. Conversely, a low available glucose level (i.e., hunger) is known to increase the activity of PeA neurons and their excitatory responses to the pedal ganglia microenvironment [3,4,6]. These opposing effects of low and high glucose level are in accordance with the hypothesis that direct glucose signaling plays a role in mediating hunger-related behavioral state [4,37]. We do not know whether PeA neurons express glucoreceptors or whether they are sensitive to the secreted insulin-like hormone. Therefore, the impact of glucose on behavioral-state related properties of serotonergic neurons is not completely defined, yet should not be overlooked.

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Conflict of Interest

All the authors declare that they have no conflict of interest.

Author Contributions

V. D. and D. S. directed the entire project. V. D. and T. D. performed the experiments. V. D., L. H. and E. I. analyzed the data. V. D., L. H., E. I. and I. Z. co-wrote the manuscript.

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