

RESEARCH ARTICLE

The activity of isolated neurons and the modulatory state of an isolated nervous system represent a recent behavioural state

Varvara E. Dyakonova^{1,*}, Laszlo Hernádi², Etsuro Ito³, Taisia L. Dyakonova¹, Ilya A. Chistopolsky¹, Igor S. Zakharov¹ and Dmitri A. Sakharov¹

ABSTRACT

Behavioural/motivational state is known to influence nearly all aspects of physiology and behaviour. The cellular basis of behavioural state control is only partially understood. Our investigation, performed on the pond snail *Lymnaea stagnalis* whose nervous system is useful for work on completely isolated neurons, provided several results related to this problem. First, we demonstrated that the behavioural state can produce long-term changes in individual neurons that persist even after neuron isolation from the nervous system. Specifically, we found that pedal serotonergic neurons that control locomotion show higher activity and lower membrane potential after being isolated from the nervous systems of hungry animals. Second, we showed that the modulatory state (the chemical neuroactive microenvironment of the central ganglia) changes in accordance with the nutritional state of an animal and produces predicted changes in single isolated locomotor neurons. Third, we report that observed hunger-induced effects can be explained by the increased synthesis of serotonin in pedal serotonergic neurons, which has an impact on the electrical activity of isolated serotonergic neurons and the intensity of extrasynaptic serotonin release from the pedal ganglia.

KEY WORDS: Serotonergic neuron, Extrasynaptic release, Volume transmission, Neuromodulation, *Lymnaea stagnalis*, Neurotransmitter synthesis

INTRODUCTION

Behavioural states defined as specific coordinated modes of behavioural, neuronal, metabolic, sensory and cognitive activities can be distinguished in both simple organisms and higher order animals. Knowledge of the cellular mechanisms underlying a behavioural state is constantly increasing (Maimon, 2011; Palmer and Kristan, 2011; Bargmann, 2012; Dyakonova, 2014). However, it is still unclear whether a behavioural state produces long-term transformations of individual neurons that can be preserved after neuronal isolation and if so, by what means.

Non-synaptic (volume or extrasynaptic) transmission of signal molecules has been suggested as a possible mechanism for behavioural state-related changes at the cellular level (Dash et al., 2009; Bargmann, 2012; Donnelly et al., 2013; Dyakonova, 2014). It is a widespread mode of intercellular communication that occurs in the extracellular space with signal molecules moving via diffusion and convection (Sakharov et al., 1990b; Agnati et al., 2006). This

mechanism allows for general information to be widely spread among potential receiving cells belonging to functionally different groups and is linked to the so-called 'neuromodulatory state', which is believed to play a key role in defining functional configurations of neuronal ensembles. Changes in the content of signal molecules have been demonstrated to correlate with certain behavioural states (Glover and Lent, 1991; Lent et al., 1991; Adamo et al., 1995; Dash et al., 2009). Moreover, specific changes in extracellular neurotransmitter contents achieved by pharmacological means were reported to, at least in part, imitate a specific behavioural state (Horvitz et al., 1982; Dyakonova and Sakharov, 1995; Stevenson et al., 2005; Donnelly et al., 2013).

The role of serotonin in mediating a hunger/feeding behavioural state has been suggested and proved in many invertebrates (Horvitz et al., 1982; Lent and Dickinson, 1988; Sakharov, 1990a; Sakharov, 1990b; Palmer and Kristan, 2011; Donnelly et al., 2013), including molluscs (Alania et al., 2004; Hernádi et al., 2004; Gillette, 2006; Hirayama and Gillette, 2012; Hirayama et al., 2014; Dyakonova, 2014). In this study, we utilized the advantages of the mollusc *Lymnaea stagnalis* to elucidate how a hunger behavioural state is represented at a single serotonergic cellular level. For this reason, we selected the serotonergic neurons, specifically cerebral giant cells (CGCs) and Pedal A (PeA) cells, as model cellular systems for the present investigations. The paired cerebral CGC neurons are modulators of the feeding central pattern generator (McCrohan and Audesirk, 1987; Yeoman et al., 1994), whereas the neurons belonging to the PeA cluster of the pedal ganglia directly control locomotion (Syed and Winlow, 1989), which is affected by feeding-related behavioural states.

First, we aimed to compare the electrical properties of identified neurons isolated from hungry and fed animals to see whether the behavioural state produces long-term changes that are preserved even after complete isolation of a neuron. Second, we aimed to examine whether the chemical neuroactive microenvironment of pedal ganglia is affected by changes in nutritional state, and whether any observed differences are essential to induce predictable changes in the isolated neurons. We observed higher activity in isolated hungry neurons and a higher excitatory influence of the extrasynaptic release of neuroactive substances from the hungry pedal ganglia. We suggest that these effects can be explained, at least in part, by increased serotonin synthesis and release within the studied serotonergic neurons.

RESULTS

Electrical activity of CGC and PeA neurons in isolated central ganglia from fed and food-deprived snails

In the central nervous system (CNS) preparations taken from food-deprived (24 h) snails, the PeA (Fig. 1A) and CGC (Fig. 2A) serotonergic neurons showed significantly enhanced firing rate (Figs 1B,C and 2B,C) and a tendency to depolarize compared with the fed control (Figs 1D and 2D).

¹Institute of Developmental Biology, Russian Academy of Sciences, Moscow 119334, Russia. ²Balaton Limnological Institute, MTA Centre for Ecological Research, Tihany H-8237, Hungary. ³Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, Shido, Sanuki 769-2193, Japan.

*Author for correspondence (dyakonova.varvara@gmail.com)

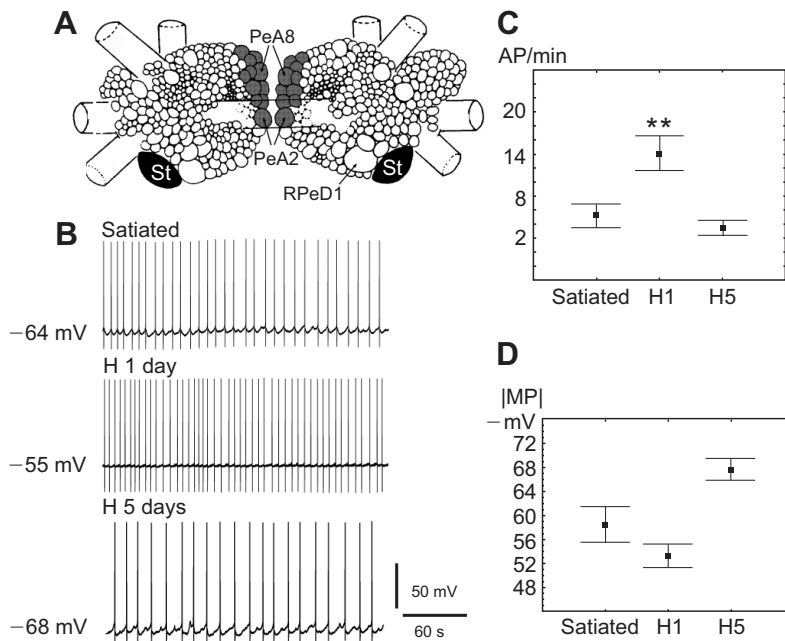


Fig. 1. The effect of food deprivation for 1 and 5 days on the activity of PeA neurons in an isolated CNS. (A) The positions of the PeA cluster (shaded), PeA2, PeA8 and RPeD1 neurons (arrows) at the dorsal surface of the paired pedal ganglia of *Lymnaea stagnalis*. St, statocyst. Modified from Slade et al., 1981. (B) Records of PeA8 cell electrical activity in isolated central ganglia from satiated and food-deprived for 1 day (H 1 day) and 5 days (H 5 days) snails. (C) Mean frequency of action potentials per minute (AP min⁻¹) of neurons belonging to the PeA cluster. Snails were fed, food-deprived for 1 day ($N=20$, $P<0.005$) and food-deprived for 5 days ($N=24$, n.s.). (D) Mean membrane potential of neurons belonging to the PeA cluster. Snails were fed, food-deprived for 1 day ($P=0.07$) and food-deprived for 5 days (n.s.). Kruskal–Wallis ANOVA test. All values are means \pm s.e.

In the preparations taken from starved snails (5 days), no significant difference in the firing rate (Fig. 1C) and membrane potential (Fig. 1D) of PeA neurons was observed in comparison with the fed control. In the CGC cells, the firing rate was significantly lower (Fig. 2B,C) and the membrane potential significantly hyperpolarized than in the fed control (Fig. 2D).

In an additional experimental series, the effect of 18 h and 36–48 h food deprivation on the electrical activity of PeA cells was investigated (not illustrated). An increased firing rate was observed after 18 h of food deprivation (24 ± 2 spikes min⁻¹ versus fed 13.5 ± 3 spikes min⁻¹, $P<0.01$, $N=34$). The increased firing rate was maintained in preparations obtained after 36–48 h of food deprivation (20 ± 2 spikes min⁻¹ versus fed 9 ± 1 spikes min⁻¹, $P<0.01$, $N=34$). Additionally, the membrane potential of the PeA cells was depolarized compared with the fed control (54 ± 1.5 mV versus fed 59 ± 1.5 mV, $P<0.05$). The activity of control and hungry

neurons was a little higher in the second set of experiments in comparison with the first experimental series. One possible explanation for this difference is that the second set of experiments was performed in a different season.

To exclude the possibility that food deprivation produces unspecific activation of neurons in general, we used an identified dopaminergic neuron (RPeD1) as a control. The neuron is located in the right pedal ganglion (Fig. 1A) and is involved in the regulation of respiratory behaviour (Haydon and Winlow, 1981; Syed et al., 1990; Syed and Winlow, 1991; Taylor and Lukowiak, 2000). No significant differences in the firing rate and membrane potential were observed between RPeD1 cells obtained from satiated or starved (1 or 5 days) snails. The membrane potential was 66 ± 2 mV in fed animals, 66 ± 3 mV in 1-day food-deprived ($n=8$) and 63 ± 2 mV in 5-day food-deprived snails ($N=8$). Electrical activity was observed only in one cell from all experiments (in 5-day food-deprived snails).

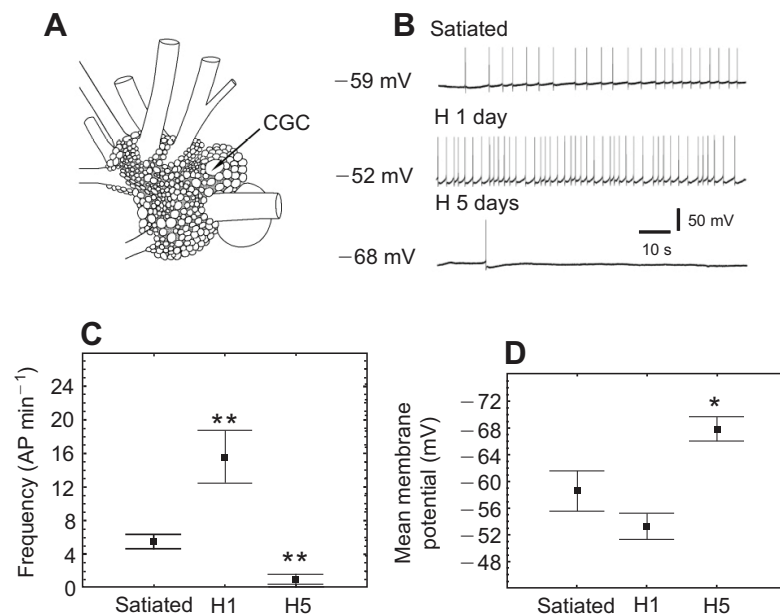


Fig. 2. The effect of food deprivation for 1 and 5 days on the activity of CGC neurons in an isolated CNS. (A) The position of the CGC neuron (arrow) at the ventral surface of the right cerebral ganglion of *Lymnaea stagnalis*. (B) Records of CGC cell electrical activity in isolated central ganglia from satiated and food-deprived for 1 day (H 1 day) and food-deprived for 5 days (H 5 days) snails. (C) Mean frequency of action potentials per minute (AP min⁻¹) of CGC neurons. Left to right: fed, food-deprived for 1 day ($N=14$, $P<0.005$) and food-deprived for 5 days ($N=15$, $P<0.005$). (D) Mean membrane potential of CGC neurons. Left to right: fed, food-deprived for 1 day (n.s.) and food-deprived for 5 days ($P<0.05$). Kruskal–Wallis ANOVA test. All values are means \pm s.e.

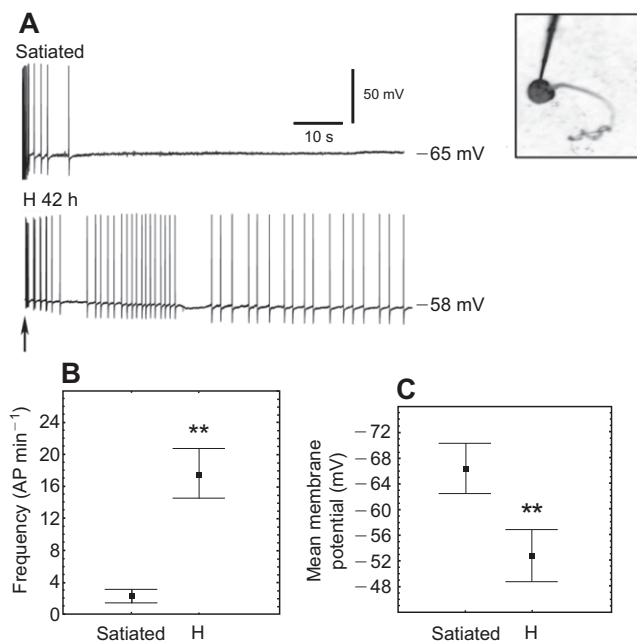


Fig. 3. The effect of food-deprivation for 24–42 h on the activity of isolated PeA neurons. (A) Records of electrical activity of isolated PeA cells from satiated snails and from snails food deprived for 42 h (H 42 h). An arrow indicates the moment of complete cell isolation. The records were taken in one experiment; the real time difference between upper and low trace is 3 min. Modified photo of an isolated PeA cell on an electrode. (B) Mean frequency of action potentials per minute (AP min^{-1}) of isolated neurons belonging to the PeA cluster [satiated and food-deprived (H), $N=23$, $P<0.007$]. (C) Mean membrane potential of neurons belonging to the PeA cluster [satiated and food-deprived (H), $N=23$, $P<0.01$]. Kruskal–Wallis ANOVA test. All values are means \pm s.e.

Electrical activity of PeA neurons isolated from fed and food-deprived snails

When we isolated PeA neurons from the ganglia of satiated animals or snails unfed for 24–42 h and kept them in the same standard snail saline buffer, the electrical differences between cells from satiated and hungry snails were preserved for at least 20–30 min following their isolation. The rate of firing of neurons in hungry snails was significantly higher ($N=23$, $18 \pm 3 \text{ spikes min}^{-1}$ versus satiated $3.1 \pm 1 \text{ spikes min}^{-1}$, $P<0.007$, Fig. 3A,B). Additionally, the neurons isolated from starved specimens had a more depolarized membrane potential than those taken from satiated animals ($53 \pm 4 \text{ mV}$ versus satiated $67 \pm 4 \text{ mV}$, $P<0.01$, $n=23$, Fig. 3C). Therefore, these data indicate that hunger as a behavioural state is manifested in the electrical properties of individual PeA neurons and was maintained even after their complete isolation.

Extrasynaptic release from pedal A cluster neurons of food-deprived and fed snails

To test whether food deprivation causes any change in the extrasynaptic neurochemical content, we applied a movable biosensor (PeA neuron isolated from a different preparation, Fig. 4). The responses of the biosensor placed near the PeA cluster of pedal ganglia of hungry (36 h) and satiated snails were compared ($N=20$). Measurements were repeated and verified with the use of a new biosensor.

In all cases ($N=56$) the biosensors measured excitation near the PeA neuronal cluster of either hungry or satiated snails. In eight of 10 experiments the excitatory effect of the neurochemical environment

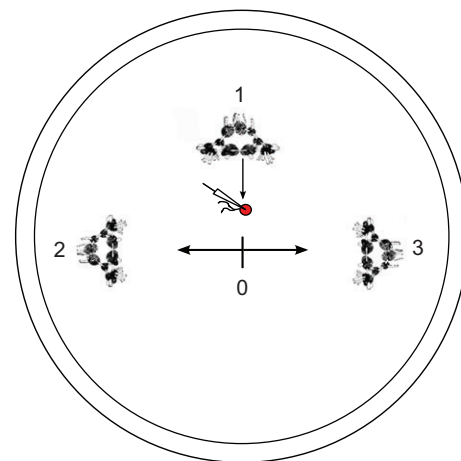


Fig. 4. A schematic representation of the experimental procedure used for the investigation of the modulatory state of ganglia isolated from fed and food-deprived snails. Three preparations of central ganglia were used in one experiment and placed in the same dish with a distance between preparations of approximately 1 cm. The first nervous system (marked as 1) was used as a source of isolated neurons (biosensors). The second and the third preparations (marked as 2 and 3) were taken from the fed and food-deprived snails. Their left–right positions were altered in the experiments and the experimenter was not aware where the fed and food-deprived preparations were placed. The connective tissue sheath was removed from the pedal ganglia. The isolated neuron impaled with the microelectrode was moved away from the pedal ganglion of the first preparation and placed at the ‘zero’ point (0) in the middle between the hungry and satiated pedal ganglia for 2 min. Then it was moved to the pedal cluster of preparation 2 at the distance less than half-cell size ($20\text{--}25 \mu\text{m}$) for 2 min, placed back at the zero point (2 min) and then moved to the PeA cluster of preparation 3 (2 min). This procedure was repeated several times in one experiment. In each position the electrical activity of the biosensor was measured. In each experiment two to three biosensors were used to check the concordance in their responses.

of the pedal ganglia from hungry snails was significantly stronger (more than 20%) on all biosensors used in these experiments ($N=48$). In only one experiment we observed that the effect of satiated preparation was stronger than the effect produced by ganglia from hungry animals, and in only one other experiment we found that the difference in the biosensor response to the hungry and satiated PeA cluster was not statistically significant (less than 5%). A paired Wilcoxon test indicated a significant difference between the effects produced by the extrasynaptic environment of hungry and satiated pedal ganglia ($P=0.02$, $Z=2.2$, Fig. 5A,B). These findings indicate that hunger, as a behavioural state, is able to affect the extrasynaptic release of neuroactive compounds from neurons of the ganglia.

In an additional experimental series we investigated whether the observed extrasynaptic release of neuroactive compounds might have a neurohormonal role in addition to an earlier reported role in the cooperation of neuronal activity within the PeA cluster or the pedal ganglia (Chistopol’skii and Sakharov, 2003). The only barrier between the ganglia and the hemolymph in *Lymnaea* is formed by the connective tissues around the ganglia. We tested whether the biosensor is able to detect the volume release of the pedal ganglia in the presence of intact connective tissues. In all cases ($N=8$), the excitatory response of a biosensor was evident (Fig. 6), although it was lower than in the preparations that were free of the connective tissue sheath. These data indicate that some substances of the central ganglia liberated for volume communication might also be involved in neurohormonal communication.

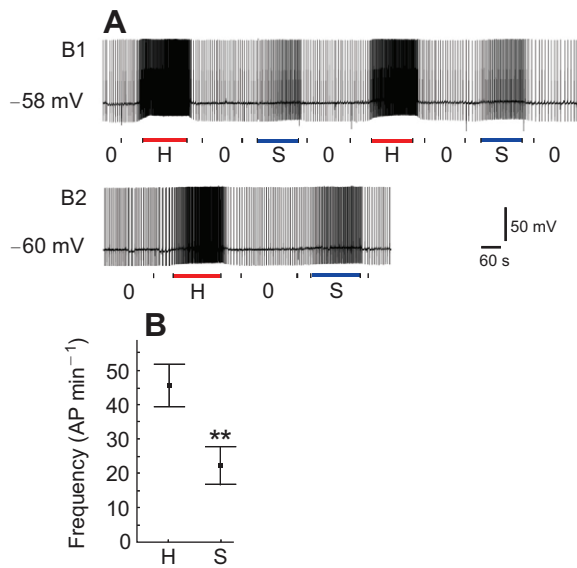


Fig. 5. The modulatory state (extrasynaptic neurotransmission) of pedal ganglia taken from fed and food-deprived snails studied by a movable biosensor (isolated PeA neuron). (A) Activity of two biosensors (marked as B1 and B2) of one experiment at the zero point (0), near the PeA cluster of pedal ganglia taken from the hungry ('H', red line) and satiated ('S', blue line) snails. The vertical lines mark the start of the biosensor movement. (B) Mean difference in frequency of action potentials per minute (AP min^{-1}) of biosensors between the 'zero point' and the nearby hungry PeA cluster (H), and between 'zero point' and the nearby satiated PeA cluster (S). ** $P < 0.01$, Kruskal–Wallis ANOVA test. All values are means \pm s.e.

Serotonin content in the pedal and buccal ganglia taken from fed and food-deprived snails

The electrical activity of PeA neurons (*in situ* and in isolation) as well as the excitatory effect of extrasynaptic neurotransmitter release from the pedal ganglia have been demonstrated to be strongly dependent upon the rate of serotonin synthesis (Kabotyanski et al., 1991; Dyakonova and Sakharov, 2001a,b; Chistopol'skii and Sakharov, 2003; Dyakonova et al., 2009). To test whether enhanced serotonin synthesis may underlie at least part of the observed differences between hungry and satiated preparations, we measured the serotonin content in the pedal ganglia taken from satiated and food-deprived snails. We also tested the serotonin content in the buccal ganglia, where serotonin is released by the axon of the CGCs, which are the feeding modulators (McCrohan and Audesirk, 1987; Yeoman et al., 1994).

High-performance liquid chromatography (HPLC) demonstrated a significant increase of the serotonin content in the pedal ganglia taken from hungry snails (Fig. 7A). At 12 and 48 h of food deprivation, the difference in serotonin content was significant [$132.4 \pm 6.3 \text{ pmol mg}^{-1}$ ($N=5$) and $114.8 \pm 8 \text{ pmol mg}^{-1}$ ($N=5$) versus fed $89 \pm 12.9 \text{ pmol mg}^{-1}$ ($N=5$)]. Similarly, an increase in the serotonin content was observed in the buccal ganglia of food-deprived snails [$8.9 \pm 1.1 \text{ pmol mg}^{-1}$ ($N=5$) and $7.9 \pm 0.8 \text{ pmol mg}^{-1}$ ($N=5$) versus fed $5.3 \pm 2.1 \text{ pmol mg}^{-1}$ ($N=5$) Fig. 7B]. In contrast, the dopamine (DA) content changed insignificantly during these periods of food deprivation in both the ganglia. Our present findings show that the increased serotonin content of pedal ganglia correlated with the increased firing rate of PeA neurons are consistent with our hypothesis that differences in the rate of serotonin synthesis may at least partially explain the differences in the firing rate and extrasynaptic neurotransmitter release between satiated and hungry neurons.

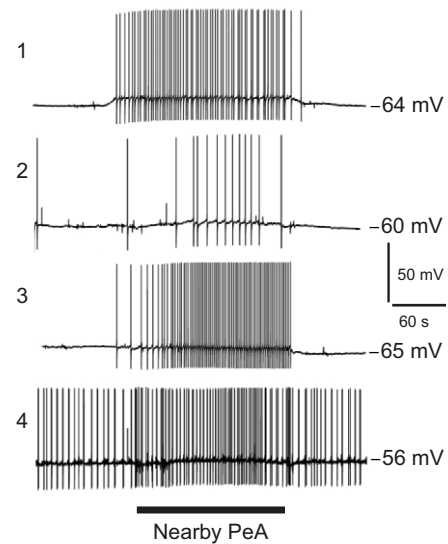


Fig. 6. The responses of biosensors in four different experiments (1 to 4) to a nearby PeA cluster of pedal ganglia with intact connective tissues. The dark horizontal line indicates the biosensor position near the PeA cluster of pedal ganglia. It corresponds to the unmovable state of a biosensor. In all cases the excitatory effect is evident.

DISCUSSION

The mechanisms of behavioural state control at the single-cell level are still only partially understood. Our investigation, performed on the pond snail *Lymnaea stagnalis* whose nervous system is useful for work on completely isolated neurons, provided several results related to this problem. First, we demonstrated that the behavioural state can produce long-term changes in individual neurons that persist even after neuron isolation from the nervous system. Specifically, we found that pedal serotonergic neurons that control locomotion show higher activity and lower membrane potential after being isolated from the nervous systems of food-deprived animals. Second, we showed that the chemical neuroactive microenvironment of the pedal ganglia changes in accordance with the nutritional state of an animal and produces predictable changes in single isolated serotonergic neurons placed in its vicinity. Third, we report that observed hunger-induced effects can be explained by the increased synthesis of serotonin in pedal serotonergic neurons.

Storage of the behavioural state in completely isolated neurons

The storage of the nutritional state in an isolated nervous system has been demonstrated earlier in *Lymnaea* (Elliott and Andrew, 1991; Hernádi et al., 2004) and other molluscs (Gillette, 2006; Hirayama and Gillette, 2012; Hirayama et al., 2014). The results of the present study agree with these previous studies and show that 24–36 h of food deprivation causes an excitation of CGC and PeA serotonergic neurons in intact ganglia (isolated CNS). In addition, we found that a longer hunger experience (5–6 days of food deprivation) had no such effect on the examined neurons and that in CGC neurons, the opposite effect was observed after 5 days of hunger. Notably, opposite effects after 1 day and 5 days of food deprivation on aversive learning were found in behavioural experiments in the same animal (Mita et al., 2014).

Here, we demonstrate for the first time that the behavioural state is represented in the electrical activity of a single completely isolated neuron. Specifically, differences in the firing rate and the

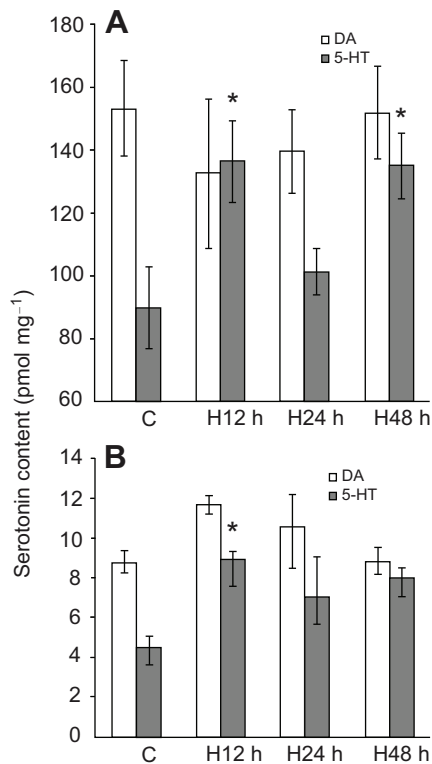


Fig. 7. The effect of food deprivation on the serotonin content in the pedal and buccal ganglia of *Lymnaea*. The results of the HPLC analysis of serotonin (5-HT) and dopamine (DA) content in pedal (A) and buccal (B) neurons from control (C, satiated) and 12–48 h food-deprived snails. * $P < 0.05$, Kruskal–Wallis ANOVA test. All values are means \pm s.e.

membrane potential were found in PeA serotonergic neurons isolated from hungry and satiated snails. These cells are known to deliver serotonin to various locomotor organs of *Lymnaea stagnalis*, specifically the ciliated epithelium of the sole and locomotor muscles (Syed and Winlow, 1989). Studies on neurons homologous to PeA neurons in the swimming marine gastropods *Clione limacina* and *Aplysia fasciata* have shown that strong, long-lasting excitation of these pedal serotonergic neurons is correlated with locomotor arousal (Kabotyanski et al., 1990; Kabotyanski and Sakharov, 1991; Satterlie, 1995). Locomotor arousal is associated with moderate hunger and food searching behaviour in many animals, including *Lymnaea* (Dyakonova and Sakharov, 1995). Therefore, the observed excitation in hungry serotonergic neurons agrees with their role during hunger behaviour.

Extrasynaptic volume transmission is affected by the actual behavioural state

There is now growing evidence that extrasynaptic neurotransmitter release does play an important role in interneuronal communication in the mammalian brain (Bunin and Wightman, 1998; Jaffe et al., 1998; Sem'yanov, 2005; Vizi et al., 2004; Chen et al., 2008) and in invertebrates of various taxa, such as leeches, insects, nematodes and molluscs (Bruns and Jahn, 1995; Chen et al., 1995; Spencer et al., 2000; De-Miguel and Trueta, 2005). Extrasynaptic release of serotonin and other substances from the pedal ganglia of *Lymnaea stagnalis*, shown by pharmacological experiments and the use of biosensors in our laboratory, was suggested to play a role in the cooperation of PeA cells at the level of cell bodies (Chistopol'skii and Sakharov, 2003; Dyakonova et al., 2009).

The novel finding presented here is that volume transmission depends upon the nutritional state of an animal. Biosensors detected a significant difference in the excitatory effect of different extracellular environments produced by pedal ganglia from satiated and hungry snails. PeA cells isolated from fed preparations demonstrated higher excitation when placed near the pedal A cluster neurons of the pedal ganglia taken from food-deprived snails. This effect agrees with the higher activity of PeA neurons in CNS of food-deprived snails. It is likely that extrasynaptic release is involved in a positive feedback loop that ensures the cooperative excitation of neurons including the PeA cluster neurons, which contribute to the increased behavioural arousal during hunger.

Molluscs have open blood vascular systems, which means that in some circumstances neuronal extrasynaptic neurotransmitter secretion may play an additional, neurohormonal role. Indeed, we have found significant effects of extrasynaptic release on biosensors when the natural barrier (connective tissues) between the nervous system and the hemolymph remained intact. This finding shows that neuroactive substances released by the ganglia are able to pass the barrier and reach the hemolymph. Therefore, serotonin released by the PeA locomotor neurons may potentially affect numerous targets beyond the pedal ganglia and foot musculature.

The crucial role of neuromodulation in behavioural choice and underlying neuronal mechanisms has been demonstrated in many studies (Bargmann, 2012; Dyakonova, 2014). Our results reveal that changes in the modulatory state are associated with different behavioural states and link neuromodulation with extrasynaptic neurotransmission.

Increased synthesis of serotonin may underlie the effects of the behavioural state on volume release and isolated cell activity

The increase in the rate of neurotransmitter synthesis is generally considered a compensatory response to enhanced electrical activity of neurons. However, in previous work, we had demonstrated that opposite relationships also exist: the level of serotonin synthesis in PeA neurons has an impact on their firing rate and the intensity of extrasynaptic serotonin release (Kabotyanski et al., 1991; Dyakonova and Sakharov, 2001a; Dyakonova and Sakharov, 2001b; Chistopol'skii and Sakharov, 2003; Dyakonova et al., 2009). The inhibitor of serotonin synthesis (NSD-1015) decreased while the immediate precursor of serotonin 5-hydroxytryptophan (5-HTP) increased the activity of isolated PeA neurons and PeA neurons *in situ*. NSD-1015 as well as a serotonin receptor antagonist abolished the effect of 5-HTP on the firing of isolated neurons, supporting the role of extrasynaptic serotonin release at the single-cell level (Dyakonova et al., 2009). 5-HTP also increased the excitatory response of biosensors near the PeA cluster in CNS (Chistopol'skii and Sakharov, 2003).

In the present study, the HPLC assay showed that during hunger the serotonin levels increased in both the pedal and buccal ganglia, suggesting an increase in serotonin synthesis in the serotonergic neurons. Increased neurotransmitter level could theoretically be explained also by the decreased breakdown of the serotonin. This explanation, however, is not favoured because we observed an increased firing rate of serotonergic neurons in the same behavioural conditions. These findings imply that moderate hunger activates serotonin synthesis within serotonergic neurons by an unknown mechanism. In *Pleurobranchaea californica*, a higher serotonin content was detected within single metacerebral giant (MCG) neurons from food-derived animals (Hatcher et al., 2008), which are homologues to CGC neurons in *Lymnaea*.

All these findings suggest that the increased 5-hydroxytryptamine (5-HT) synthesis may explain the increased firing activity of isolated, PeA neurons from hungry animals. Enhanced synthesis of serotonin also explains the stronger effects of neuroactive compounds released from the pedal ganglia of food-deprived animals on the activity of the biosensors.

These data are in accordance with the idea that the long-term alterations in reception and transmission of chemical signals underlie changes in neuronal communication at the single-cell level that are usually observed after changes in the behavioural state (O'Connell and Hofmann, 2012). Recent reports demonstrating that behavioural state induced changes in receptor gene expression (Whitaker et al., 2011), neuronal response to neurotransmitters (Yeh et al., 1996; Edwards et al., 2002) and in neurotransmitter synthesis (Hatcher et al., 2008) at the single-cell level are in line with this point of view as well.

In conclusion, our results stress the principal role of chemical events in the mechanisms of controlling a behavioural state at the cellular level. They are in accordance with the two widely discussed hypotheses: (1) that non-synaptic (volume or extrasynaptic) transmission plays a significant role in the distributed effects of a behavioural state, and (2) that changes in neurotransmitter synthesis are the principal means of behavioural state-induced regulation at the single-cell level. Notably, it is difficult to classify the serotonin produced by PeA neurons according to the modern classification of neuronal signal molecules. It acts as a neurotransmitter, as extrasynaptic released neuromodulator, and potentially as a neurohormone. This example is not unique. The more we know about the modes of action of neuronal signal molecules, the more difficult it is to subdivide them into classical neurotransmitters, neuromodulators, metamodulators, neurohormones and hormones. This difficulty is not surprising, as the origin of the present classification is based on an out-of-date concept that a chemical signal is only a transmitter of electrical events in the nervous system.

MATERIALS AND METHODS

Animals

Mature specimens of *Lymnaea stagnalis* Linnaeus were taken from a breeding colony, kept in dechlorinated tap water at room temperature and fed on lettuce. In the first set of experiments, two experimental groups of animals were studied: snails kept for 1 day without food, and snails kept for 5 days without food. Each group had a related control, snails fed on lettuce.

In the second set of experiments, performed in a different season, the effect of 18 h and 36–48 h of food deprivation on the activity of PeA cells in CNS was studied. In experiments on isolated cells, snails were kept without food for 24–42 h and the water in the chambers was changed daily. The experimental and control animals were investigated in parallel in one experiment.

Electrophysiology and cell isolation

In each experiment, the central ganglia were dissected from two animals (fed and food-deprived) anaesthetized with an injection of 0.1 mmol l⁻¹ MgCl₂. The central ganglia were placed into a 2.5 mg ml⁻¹ solution of pronase E (Sigma) for 15 min, washed in a standard snail Ringer solution (50 mmol l⁻¹ NaCl, 1.6 mmol l⁻¹ KCl, 4 mmol l⁻¹ CaCl₂, 8 mmol l⁻¹ MgCl₂, 10 mmol l⁻¹ Tris; pH 7.6) and pinned with Sylgard in a 4 ml chamber with a distance of approximately 1 cm between preparations. The connective tissue sheath was then removed from the pedal ganglia.

Visual identification of the CGC and PeA2/A8 neurons was performed based on their location, size and colouration. Other neurons were randomly taken from serotonergic PeA clusters to assess whether the observed effects of hunger were common to different cluster members. The neuron that was selected for examination was impaled with a standard glass microelectrode (10–20 MΩ filled with 3 mol l⁻¹ KCl). A standard set-up

for microelectrode recording was used. The electrophysiological recordings were stored in computer files using a homemade program.

For neuron isolation, we utilized previously developed methods (Dyakonova et al., 2009). Using the intracellular microelectrode as a pull, the neuron was gently pulled out of the tissue until separation of the proximal neurite from the neuropile was achieved. The electrical activity of the cell was monitored during isolation. The cells, which demonstrated membrane injury, were not used in the experiments.

Investigation of the modulatory state of pedal ganglia taken from snails of different behavioural states

Our approach was developed on the basis of earlier used methods for detection of extrasynaptic release from the ganglia of *Lymnaea* (Chistopol'skii and Sakharov, 2003; Dyakonova and Dyakonova, 2010). Three preparations of central ganglia were used in one experiment and placed in the same chamber with a distance between preparations of approximately 1 cm (Fig. 4).

The first nervous system was used as a source of isolated neurons (biosensors) and was treated as above (the procedure of neuron isolation). The second and the third preparations were taken from fed (control) and 36 h food-deprived snails. Their positions in the chamber were altered in different experiments, and the investigator was not aware where the fed and food-deprived preparations were placed ('blind procedure'). The connective tissue sheath was removed from the pedal ganglia.

The isolated neuron impaled with the microelectrode was moved away from the pedal ganglion of the first preparation and placed at a 'zero' point in the middle between the hungry and satiated pedal ganglia for 2 min (Fig. 4). Then, it was moved to the pedal cluster of the second experimental preparation at a distance less than half-cell size (20–25 μm) and kept in this position for 2 min, then placed back at the zero point (2 min) and moved to the PeA cluster of the third preparation ganglia (2 min). This procedure was repeated several times in one experiment. In each position the electrical activity of the biosensor was measured. In each experiment, two to three biosensors were used to check the concordance in their responses. Ten experiments were performed in total.

HPLC assay of monoamines

Monoamines (5-HT, DA) were measured from the pedal and buccal ganglia of fed and starved animals. Animals were starved for 12, 24 and 48 h. After removing the shell, the pedal and buccal ganglia were dissected and homogenized in 0.1 mol l⁻¹ perchloric acid containing isoprenaline (1 pmol μl⁻¹) as internal standard and were centrifuged. Pedal ganglia were homogenized in 1000 μl, whereas buccal ganglia were homogenized in 500 μl volume.

The monoamines were assayed by a Waters high-performance liquid chromatograph equipped with an electrochemical detector and an integrator unit. Aliquots of the clear supernatant were injected into a reverse phase C18 Nucleosil column. The mobile phase contained 0.05 mol l⁻¹ sodium acetate, 0.2 mol l⁻¹ EDTA, 1 mmol l⁻¹ octanesulfonic acid, 10% methanol and the final pH was adjusted to 4.0 with citric acid. The flow rate was 1.0 ml min⁻¹ and the column temperature was 24°C. The system identified the DA and 5-HT peaks on the chromatogram by the injection of known amounts of DA and 5-HT into the reverse phase prior to the injection of samples. The exact amounts of DA and 5-HT were calculated automatically by the integrator unit of the system.

Data analysis

The significance of differences was tested either by non-parametric Kruskal–Wallis ANOVA test (the differences in spike frequency and membrane potential between hungry and fed neurons *in situ* and isolation; HPLC data analysis) or by the paired Wilcoxon signed-rank test for dependent samples (the differences in biosensor activity near fed and food-deprived ganglia) using the STATISTICA program (StatSoft Inc., 1993). All values are given as means with the standard error and level of significance.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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