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Coordination of rhythm-generating units via NO and extrasynaptic neurotransmitter release

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Abstract The buccal ganglia of the mollusc, *Lymnaea stagnalis*, contain two distinct but interacting rhythm-generating units: the central pattern generator for the buccal rhythm and nitrergic B2 neurons controlling gut motility. Nitric oxide (NO) has previously been demonstrated to be involved in the activation of the buccal rhythm. Here, we found that NO-generating substances (SNP and SNAP) activated the buccal rhythm while slowing the endogenous rhythm of B2 bursters. The inhibitor of NO-synthase, L-NNA, the NO scavenger PTIO, or the inhibitor of soluble guanylyl cyclase, ODQ, each produced opposite, depolarising effects on the B2 neuron. In isolated B2 cells, only depolarising effects of substances interfering with NO production or function (PTIO, L-NNA and ODQ) were detected, whereas the NO donors had no hyperpolarising effects. However, when an isolated B2 cell was placed close to its initial position in the ganglion, hyperpolarising effects could be obtained with NO donors. This indicates that extrasynaptic release of some unidentified factor(s) mediates the hyperpolarising effects of NO donors on the B2 bursters. The results suggest that NO is involved in coordination between the radula and foregut movements and that the effects of NO are partially mediated by the volume chemical neurotransmission of as yet unknown origin.

Keywords CPG · Extrasynaptic transmission · Volume transmission · Nitrergic neuron · Mollusc

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Abbreviations

L-NNA	N-Nitro-l-arginine
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PTIO	2-Phenyl-4,4,5,5-tetramethylimidazonine-l-oxyl-3-oxide
SNAP	*s*-Nitroso-*N*-acetylpenicillamine
SNP	Sodium nitroprusside

Introduction

Interactions among rhythmically active neuronal circuits are important for generating complex behaviours, yet little is known about their underlying cellular mechanisms. We addressed this issue in the *Lymnaea stagnalis* buccal ganglia, which contain two distinct but interacting rhythm-generating units. Firstly, they have a well-characterised central pattern generator for the buccal (radula) rhythm (Benjamin and Rose 1979; Elliott and Susswein 2002) and secondly, they contain neurons that have been identified as controlling gut motility (Perry et al. 1998). The latter are a bilaterally symmetrical pair of NO-synthesising neurons called B2 cells, which innervate the foregut and directly modulate its motility (Perry et al. 1998; Sadamoto et al. 1998; Moroz et al. 2005). The B2 cells receive excitatory input from the central pattern generator during the second phase of the buccal rhythm (Benjamin and Rose 1979; Park et al. 1998) and also demonstrate "fast" bursting rhythm that is independent of and more frequent than the buccal rhythms (Benjamin and Rose 1979).

The role of NO in chemical sensory activation of the buccal central pattern generator has been well documented in electrophysiological, behavioural and genetic studies

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(Moroz et al. 1993; Elphick et al. 1995; Sadamoto et al. 1998; Korneev et al. 2002). Whether NO is also capable of affecting the B2 burster, thus controlling gut motility, remains unknown. It is intriguing that the B2 is a NO-synthesising neuron itself, and a number of principal interneurons of the buccal central pattern generator are located in close vicinity to the B2 soma (Korneev et al. 1998; Park et al. 1998; Sadamoto et al. 1998; Staras et al. 1998). Experiments using NO-sensitive microelectrodes suggested that NO is released from the soma of the B2 neurons (Kobayashi et al. 2000). This release corresponds to the bursting activity of the B2 cell and theoretically may affect the surrounding neurons.

We started the elucidation of the complex interplay between NO and two independent but transitory phase-locked generators, one of which produces NO by itself, in order to answer the question of whether and how NO affects and coordinates the B2 rhythm with the activation of the buccal central pattern generator. Part of the preliminary data was published in symposium material (Dyakonova and Dyakonova 2008a) and as a brief communication in Russian (Dyakonova and Dyakonova 2008b).

Materials and methods

Mature specimens of *Lymnaea stagnalis* were taken from a breeding colony kept in dechlorinated tap water at room temperature and fed on lettuce. The central ganglia were dissected from an animal anaesthetised with an injection of 0.1 mM $MgCl_2$, placed into a 2.5 mg/ml solution of pronase E (Sigma) for 15 min, washed in standard snail Ringer (50 mM NaCl, 1.6 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 10 mM Tris, pH 7.6) and pinned down to Sylgard in a 4-ml chamber. The connective tissue sheath was then removed from the buccal ganglia, and the preparation was kept undisturbed for 1 h prior to use in experiments.

To monitor buccal central pattern generator activity, the B4 and B4Cl motoneurons were used, which demonstrate all three phases of the standard buccal rhythm (Staras et al. 1998). Visual identification of the B2, the B4 and B4Cl neurons was performed based on their location and size according to Benjamin and Rose (1979). The neurons were impaled with a standard glass microelectrode (10–20 MΩ, 3 M KCl). Isolation of the B2 neurons was done as in the previous studies (Dyakonova 1991; Dyakonova et al. 2009). Briefly, using the 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 isolated neuron impaled with the microelectrode was moved away from the ganglion at a distance of at least one ganglion diameter. All steps were performed in a continuous stream of Ringer's solution (0.75 ml/min). A standard set-up for microelectrode recording was used. All data were stored in computer files using the SpikeC3 program.

An inhibitor of NO-synthase, *N*-nitro-L-arginine (L-NNA, 0.5 mM), a scavenger of NO, 2-phenyl-4,4,5,5-tetramethylimidazonine-l-oxyl-3-oxide (PTIO, 0.25 mM), donors of NO, sodium nitroprusside (SNP, 0.1; 0.5 mM) and *s*-nitroso-*N*-acetylpenicillamine (SNAP, 1 mM), and a selective inhibitor of soluble guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 0.02; 0.05 mM), were all obtained from Sigma and applied to the bath by switching superfusion inflow reservoirs. Since the effectiveness of all drugs had been tested earlier in molluscan nervous systems, the applied concentrations were chosen in accordance with available data (Moroz et al. 1993, 1994; Park et al. 1998; Koh and Jacklet 1999). The significance of the differences in spike frequency prior to and after drug administration was tested by the non-parametric paired Wilcoxon signed-rank test for dependent samples using the STATISTICA program (StatSoft, Inc. 1993). All values are given as mean with standard error and level of significance.

Results

Relationships between the buccal and the B2 rhythms in isolated untreated CNS

We analysed 47 untreated preparations in which the B2 cell activity and the buccal rhythm were simultaneously recorded. The B2 cells generated their fast rhythm (Figs. 1c, 2) in 33 of 47 cases: in 14 of 15 preparations with the silent buccal central pattern generator and in 19 of 28 preparations with the active central pattern generator. The central pattern generator was considered active independently of the period of rhythm generation (from 4 s up to 60 s). This included only three preparations demonstrating fast feeding (4–10 s) and 25 preparations with a slow rhythmic activity. Slow feeding rhythms had previously been described in *Lymnaea*, they are generated by the same buccal central pattern generator and include the same three phases characteristic for the fast feeding (Elliott and Vehovszky 2000; Kemenes et al. 2001).

The B2 membrane potential was significantly higher in preparations with active buccal central pattern generator: -62.2 ± 2.6 versus -52.8 ± 2.1 in preparations with silent generator, $P < 0.05$, Kolmogorov–Smirnov test (Fig. 1a). The latter suggests that B2 activity is generally down-regulated by activation of buccal rhythm.

The pattern of B2 firing during buccal rhythm generation varied among preparations (Fig. 1b, c). Besides

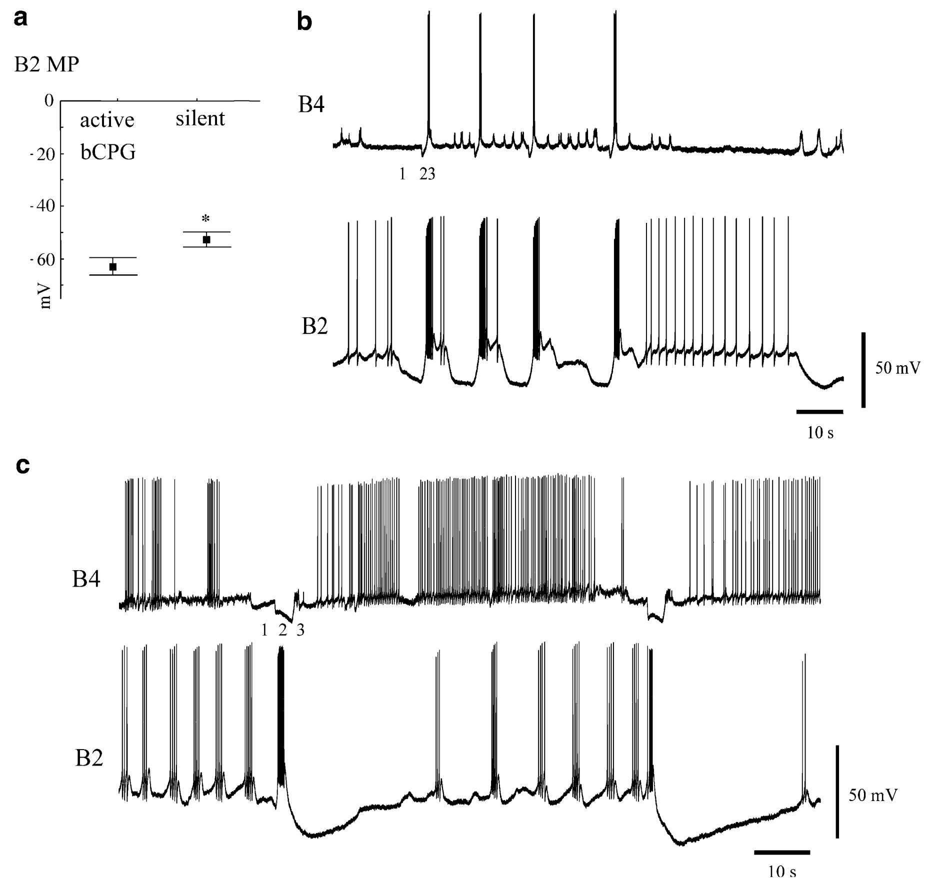

Fig. 1 Coordination of buccal central pattern generator and B2 burster in untreated CNS preparations. **a** The mean level of the B2 membrane potential (*MP*) in preparations with active buccal central pattern generator (*CPG*) versus preparations with silent buccal CPG, $*P < 0.05$, Kolmogorov–Smirnov test. **b**, **c** Various types of coordination between buccal central pattern generator and B2 activities. **b** Hyperpolarisation of the B2 neuron during period of buccal rhythm activation is interrupted by excitatory second-phase input [*MP* −60 mV (B4) and −60 mV (B2)]. **c** Two cycles of slow buccal rhythm correlate with the occurrence of giant hyperpolarising waves in the B2 [*MP* −45 mV (B4) and −70 mV (B2)]. Excitatory second-phase input is also seen. Central pattern generator activity is monitored by recording activity of B4 motoneurons everywhere. Three phases of the standard buccal cycle (*1*, *2* and *3*)

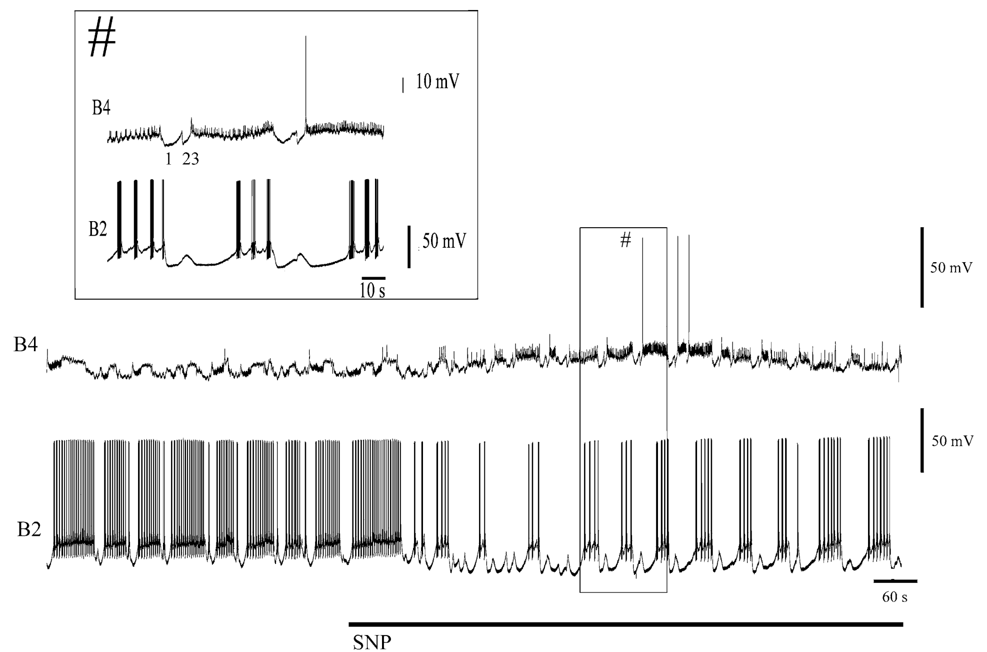

Fig. 2 Sodium nitroprusside activates buccal rhythm and simultaneously hyperpolarises the B2 burster. In the B2, SNP (SNP, 0.5 mM) increases the number of giant hyperpolarising waves, which are strongly associated with buccal cycles (see # for details). Central pattern generator activity is monitored by recording activity of B4 motoneuron. Three phases of the standard buccal cycle (*1*, *2* and *3*). [*MP* −67 mV (B4) and −71 mV (B2)]

previously reported and invariably observed excitatory input during the second phase (Benjamin and Rose 1979) (Fig. 1b, c), two other factors influenced the B2 activity pattern during feeding: tonic polarisation during the entire feeding period (Fig. 1a, b) and phase-locked giant hyperpolarising waves (Fig. 1b, c).

Giant hyperpolarising waves, are slowly developing waves that reach a maximum of −30 mV in amplitude with

duration of 60 s with no identifiable IPSPs (Fig. 1b, c), have also been previously observed in B2 cells but independently of the buccal central pattern generator activity (Benjamin and Rose 1979). Their origin remains unknown. Our analysis indicates that the occurrence of giant hyperpolarising waves correlates strongly with a slow activity of the buccal central pattern generator. Giant hyperpolarising waves were observed in only 2 of the 15 cases of silent central pattern generator, in contrast to 25 from 28 cases with active central pattern generator ($P < 0.001$, $F = 16.6$). During slow feeding, giant hyperpolarising waves occur during the third phase (Fig. 1b, c).

Our data demonstrate that there are several types of coordination between the buccal central pattern generator and the B2 activity (Fig. 1b, c). However, statistical analysis indicates that, in most cases in untreated preparations, B2 activity is down-regulated by activation of the buccal central pattern generator.

NO donors activate buccal rhythm and hyperpolarise B2 neurons

In preparations of isolated CNS with buccal ganglia, the NO donor sodium nitroprusside (SNP, 0.5 mM) activated the buccal rhythm and slowed the B2 rhythm (Fig. 2). In the B2 neuron, SNP caused tonic hyperpolarisation and activated periodical giant hyperpolarising waves, which are associated with the buccal cycles (Fig. 2, low trace). The mean frequency of the B2 cell spiking was 44 ± 8 and 17 ± 7 AP/min prior to and in 5 min of SNP application, respectively, $n = 15$, $z = 2.6$, $P < 0.01$ paired Wilcoxon signed-rank test for dependent samples (Fig. 3b).

Similar hyperpolarising effects on the B2 neuron were produced by the other NO-generating chemical SNAP (1 mM, Fig. 3a, b). SNAP activated giant hyperpolarising waves and decreased firing rate (from 58 ± 6 to 23 ± 9 AP/min, $n = 9$, $z = 2.6$, $P < 0.01$ paired Wilcoxon signed-rank test for dependent samples).

Activation of the buccal central pattern generator associated with hyperpolarisation of the B2 neurons and induction of giant hyperpolarising waves was also observed in preparations of buccal ganglia disconnected from the other parts of CNS via cutting of the cerebral-buccal connections and treated with NO donors ($n = 12$, not illustrated). Earlier similar effects were obtained with long-term stimulation of the B2 cell by depolarising current (Dyakonova and Dyakonova 2008b). These findings demonstrate that NO targets that provide NO effects on the feeding system also exist in the buccal ganglia. The effects of NO on isolated B4 motoneurons (Moroz et al. 2005; Straub et al. 2007) also support the conclusion that NO can act directly on the buccal system of *Lymnaea*.

ODQ, an inhibitor of NO-sensitive guanylyl cyclase, activates B2 neurons and decreases the hyperpolarising effects of SNP

In molluscs, as well as in mammals, a major part of the effects of NO seems to be mediated via NO-sensitive guanylyl cyclase (Huang et al. 1998; Koh and Jacklet 1999). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), whose binding site is competitive with NO, is a highly selective inhibitor of soluble guanylyl cyclase. The effectiveness of this drug has been previously demonstrated in various invertebrates, including molluscs (Koh and Jacklet, 1999). To test whether the observed effects of NO elevation are dependent upon cGMP-cyclase activity, we pre-treated the preparation with ODQ for 15–20 min.

The 0.05 mM ODQ treatment for 5 min produced depolarisation in the B2 neuron (Fig. 4a, b), and this effect increased up to the tenth minute of ODQ application. The rate of B2 firing was 27 ± 6 versus 61.4 ± 14 AP/min prior to and after ODQ treatment, respectively ($n = 10$,

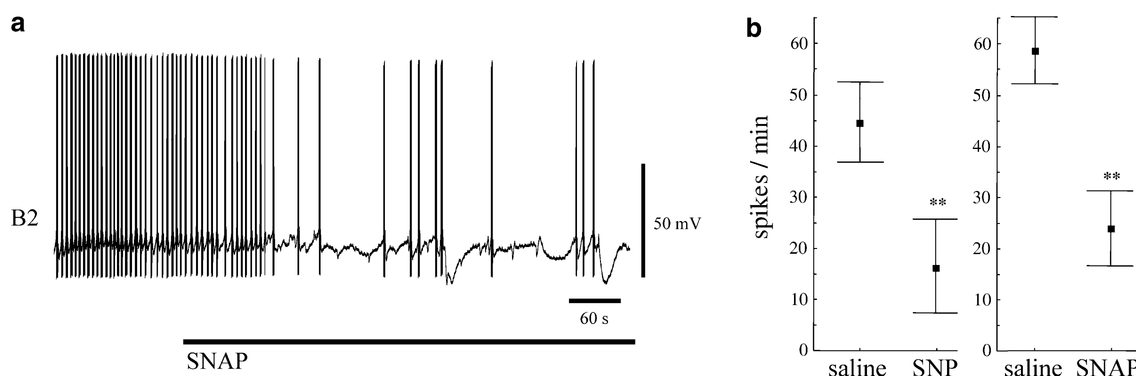

Fig. 3 The effects of NO donors on the B2 burster activity in situ. **a** The record of SNAP (0.1 mM) hyperpolarising effect on the B2 activity (*MP* −67 mV). **b** The mean firing rate of the B2 cell prior to and after 5 min of application of NO donors SNP and SNAP, firing rate is measured in spikes/min, action potentials are measured per minute, and all values are reported as mean with SEM, $^{**}P < 0.01$, the paired Wilcoxon test

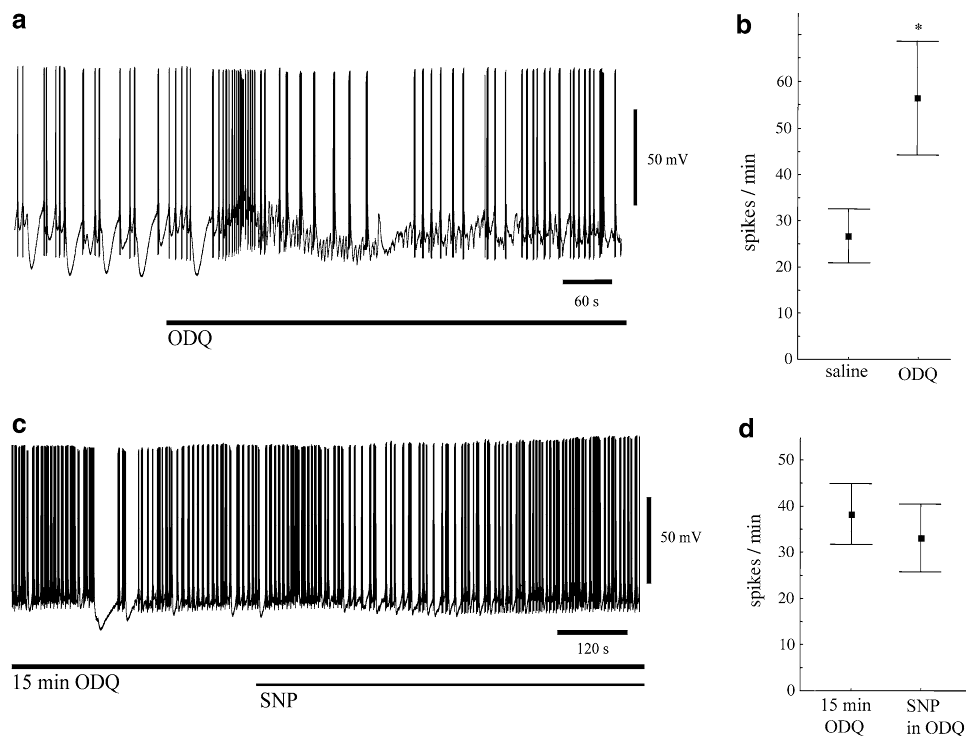

Fig. 4 The inhibitor of soluble guanylyl cyclase *ODQ* enhances the B2 neuron activity and abolishes the effect of SNP in situ. **a** The record of *ODQ* (0.05 mM) effect on the B2 activity. *ODQ* increases the B2 activity and abolishes the giant hyperpolarising waves (*MP* −67 mV). **b** The mean firing rate of the B2 neurons prior to and after 5 min of application of 0.05 mM *ODQ*, firing rate is measured in spikes/min, action potentials are measured per minute, and all values are reported as mean with SEM, $^{*}P < 0.05$, the paired Wilcoxon test. **c** The record of *SNP* (0.5 mM) effect on B2 neuron in CNS pre-treated with 0.05 mM *ODQ* for 15 min, hyperpolarising effect of SNP significantly abolished [compare with Figs. 1, 2, (*MP* −67 mV)]. **d** The mean firing rate of the B2 neurons pre-treated with ODQ for 15 min prior to and after 5 min of application of SNP in ODQ, firing rate is measured in spikes/min, action potentials are measured per minute, and all values are reported as mean with SEM, the results of paired Wilcoxon test show no difference

$P = 0.02$, $z = 2.2$, paired Wilcoxon signed-rank test for dependent samples). The effect was observed even in preparations with initially silent buccal central pattern generator, thus suggesting the existence of tonic NO release and tonic NO impact on the activity of the buccal system. In preparations that initially showed giant hyperpolarising waves, ODQ slowly decreased their occurrence; in some cases they disappeared completely (Fig. 4a).

SNP effects were significantly decreased by pre-treatment with ODQ (Fig. 4c, d). There was no significant change in the mean firing rate after SNP administration in ODQ: 35 ± 9 versus 32 ± 10 AP/min prior to and after 5 min of SNP treatment, respectively ($n = 9$, $P = 0.57$, paired Wilcoxon signed-rank test for dependent samples). This demonstrates that the observed effects of NO are at least partially mediated via activation of soluble guanylyl cyclase.

L-NNA, an inhibitor of NO-synthase, as well as the NO scavenger PTIO, activates the B2 neuron

The excitatory effects of ODQ indicated that there is a tonic impact of the nitrergic system on the activity of the buccal ganglia and on the B2 neuron. This agrees with our observation of tonic B2 activity in most preparations tested, including those with silent buccal central pattern generator. To further verify this suggestion, we tested the effects of two drugs, which decrease the NO level by different means. Specifically, we examined the effects of L-NNA, a non-specific inhibitor of NO-synthase previously tested in invertebrates, including molluscs (Huang et al. 1997; Kobayashi et al. 2000; Zayas and Trimmer 2007), and the widely used NO scavenger PTIO on the activity of the B2 cells.

L-NNA depolarised the B2 neuron and enhanced the fast B2 rhythm (Fig. 5a, b). The drug also abolished giant hyperpolarising waves in preparations where they were previously seen. The mean spiking rate was 28.3 ± 9 versus 58 ± 10 AP/min prior to and after L-NNA treatment, respectively ($n = 14$, $z = 2.9$, $P = 0.0035$, paired Wilcoxon signed-rank test for dependent samples). The effect of L-NNA could be reversed by washing out the drug (not illustrated). SNP administration in the presence of L-NNA resulted in the immediate hyperpolarisation and re-occurrence of giant hyperpolarising waves (Fig. 5a).

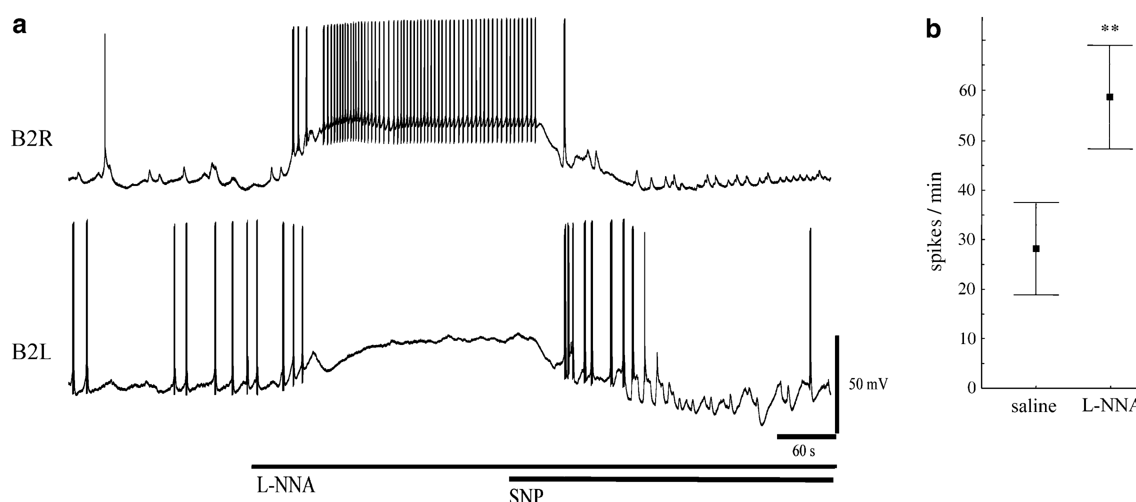

Fig. 5 L-NNA, an inhibitor of NO-synthase activates B2 neuron in situ. **a** The excitatory effect of L-NNA on the activity of B2 cells is abolished by addition of SNP to the perfusing solution [right and left B2 neurons simultaneously recorded (*MP* −55 mV and −65 mV)]. **b** The mean firing rate of the B2 neurons prior to and after 5 min of application of L-NNA 0.5 mM, firing rate is measured in spikes/min, action potentials are measured per minute, and all values are reported as mean with SEM, $*P < 0.05$, the paired Wilcoxon test

PTIO caused a similar depolarising effect reversible by washing (Fig. 6a, b). The mean spiking rate was 25 ± 10 sp/min versus 72 ± 10 AP/min prior to and after PTIO treatment ($n = 8$, $z = 2.4$, $P = 0.018$ paired Wilcoxon signed-rank test for dependent samples). Giant hyperpolarising waves could also be abolished by the drug.

The effects of both L-NNA and PTIO, which decrease the NO level by different means, were opposite to the effects of NO donors and similar to the effect of ODQ, an inhibitor of the target of NO. Their effectiveness in untreated preparations confirm the suggestion that NO is endogenously released in the nervous system of *Lymnaea* and that the level of NO controls the activity of the nitrergic neuron B2 via a negative feedback loop.

The responses of isolated B2 neurons to changes in the NO level

The data presented above indicate that NO coordinates the buccal central pattern generator and B2 activity via a reciprocal effect on these two generators and via activation of giant hyperpolarising waves in the B2 cell. We thought to determine whether the observed effects of NO on B2 activity are direct or whether they are mediated via other neurons that are sensitive to NO. Earlier, cultured B2 neurons were reported to have no response to the NO donors SNAP or DEA NONOate (Moroz et al. 2005). Here we isolated B2 cells as was done in a previous study (Dyakonova et al. 2009), and the effects of the drugs affecting the nitrergic system were examined on freshly isolated neurons.

After isolation, the B2 cell retained its characteristic fast bursting activity (Fig. 7), while the giant hyperpolarising waves could not be seen. The mean spiking rate was lower in isolated B2 neurons compared to in situ conditions (compare controls in Figs. 2b, 4b, 5b and 5e vs. the controls in Fig. 7a), and about 30% cells became silent after isolation. The mean membrane potential differed weakly in isolated and non-isolated B2 neurons −61.2 + 2.4 mV ($n = 27$) and −57.6 + 3 mV ($n = 39$), respectively.

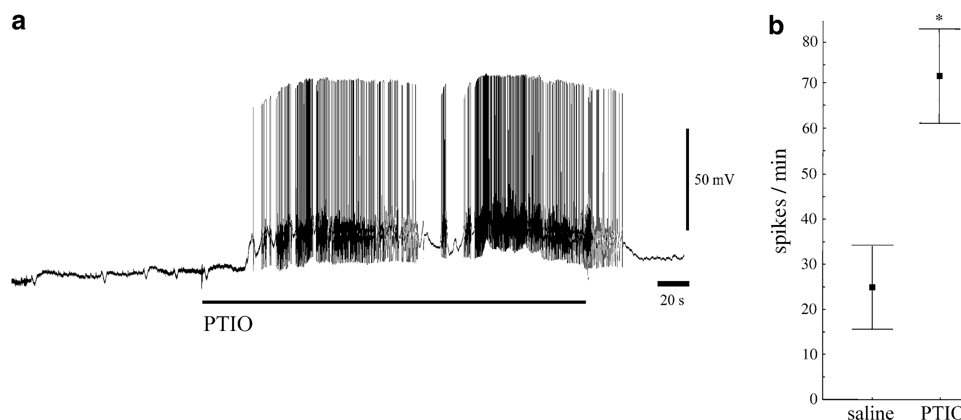

Fig. 6 The NO scavenger PTIO activates B2 neuron in situ. **a** The record of reversible excitatory effect of NO-scavenger PTIO (0.25 mM) on the B2 cell (*MP* −72 mV). **b** The mean firing rate of the B2 neurons prior to and after 5 min of application of PTIO, firing rate is measured in spikes/min, action potentials are measured per minute, and all values are reported as mean with SEM, $*P < 0.05$, the paired Wilcoxon test

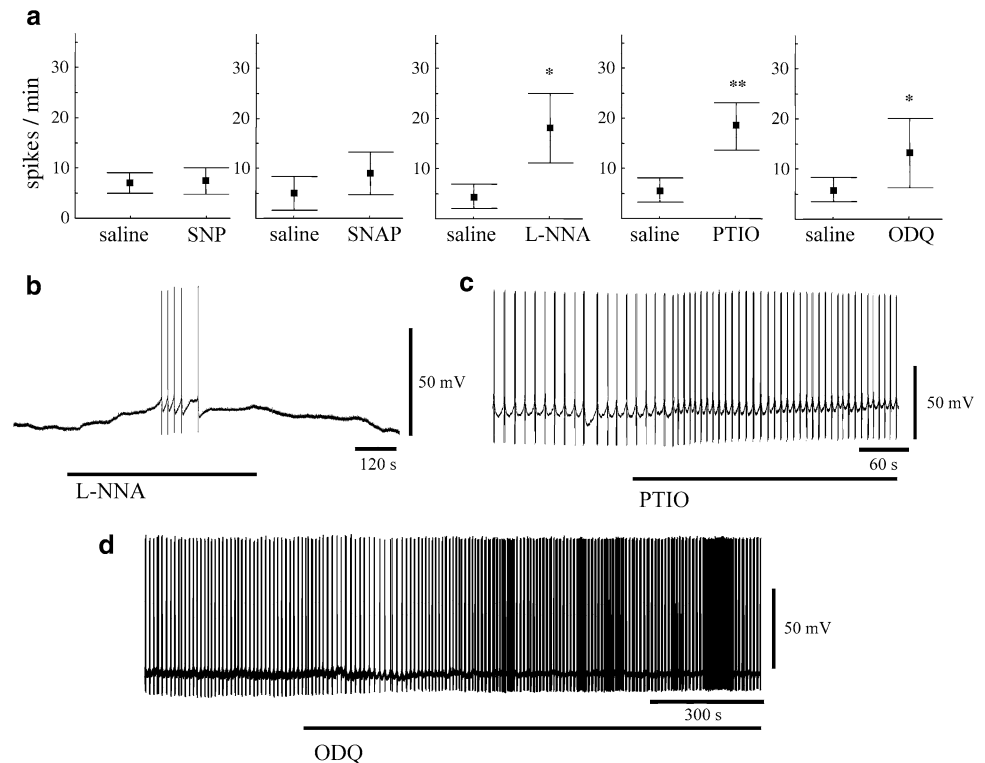

Fig. 7 Responses of isolated B2 cells to changes in the NO production and inhibition of cGMP cyclase. **a** The mean firing rate of isolated B2 prior to and after 5 min of application of *SNP*, *SNAP*, *L-NNA*, *PTIO* and *ODQ*; firing rate is measured in spikes/min, action potentials are measured per minute, and all values are reported as mean with SEM, $^{*}P < 0.05$, the paired Wilcoxon test. **b–d** The records of activity of isolated B2 prior to and after application of unspecific NO-synthase inhibitor L-NNA, 0.5 mM (**b**), the NO scavenger *PTIO* 0.25 mM (**c**) the inhibitor of cGMP cyclase *ODQ*, 0.05 mM (**d**)

The NO donors had no inhibitory effects on isolated B2 neurons (Figs. 7a, 9a): the mean spiking rate was: 7 ± 2 versus 7 ± 2.6 AP/min ($n = 8$, $P = 0.8$) prior to and after 0.5 mM SNP treatment for 5 min, and 5 ± 3 versus 8.3 ± 4.4 AP/min ($n = 9$, $P = 0.5$) prior to and after SNAP. A high concentration of SNP (2 mM) could induce an opposite, possibly non-specific, depolarising effect. Altogether, these findings confirm the data obtained in cultured B2 cells (Moroz et al. 2005).

Since isolated B2 neurons demonstrated a lower level of activity than in situ, we also tested whether the effects of the NO donors might depend upon the membrane potential level. However, NO donors did not produce significant hyperpolarising effects in cells in the subpopulation of isolated B2 neurons with initial membrane potential ranging from −38 to −56 mV ($n = 10$) or in those with −59 to −84 mV MP ($n = 7$). Isolated B2 neurons, which were depolarised by current injection (+0.2 to −0.8 nA, $n = 5$), also did not have a hyperpolarising response to SNP.

In contrast, the depolarising effects of PTIO or L-NNA, although weaker than in situ, could still be observed in isolated B2 cells (Fig. 7a–c). These drugs caused depolarisation, acceleration or initiation of bursting in initially active or silent neurons, respectively. The mean rate of spiking was 4.8 ± 2 versus 18 ± 7 AP/min prior to and after L-NNA treatment for 5 min, respectively ($n = 9$, $P = 0.02$, $z = 2.2$, paired Wilcoxon signed-rank test for dependent samples), and 5.4 ± 2 versus 18.5 ± 5 AP/min prior to and after PTIO treatment for 5 min ($n = 9$, $z = 2.5$, $P = 0.01$ paired Wilcoxon signed-rank test for dependent samples). The addition of SNP after L-NNA resulted in a weak (ca.−4 mV) hyperpolarisation in three of five cases, and in two cases, there was no effect.

The cGMP-cyclase inhibitor ODQ depolarised seven of nine isolated B2 cells (mean delta membrane potential was +2.5 ± 1.5 mV, $n = 9$). The firing rate was also significantly higher after application of ODQ: 6 ± 2 versus 13 ± 7 AP/min prior to and after ODQ treatment, respectively (Fig. 7a, d; $n = 9$, $P = 0.02$, $z = 2.2$, paired Wilcoxon signed-rank test for dependent samples). However, the effect was weaker than the effects of L-NNA and PTIO. Addition of PTIO or L-NNA to ODQ-containing Ringer resulted in a further decrease of membrane potential level and an increased firing rate of isolated B2 neurons.

These results indicate that indirect mechanisms, via interactions of B2 cells with the buccal system, underlie a major part of the effects of NO on the B2 neurons. We unexpectedly found that isolated B2 cells are more sensitive to decreases in the NO level than to increases in the NO production caused by NO donors. L-NNA, PTIO or ODQ were all capable of altering B2 activity in contrast to NO donors. Comparison of ODQ effects with those of L-NNA and PTIO suggests that there might be other targets for NO in the B2 cells besides the cGMP cyclase, or, alternatively, that some of the depolarising effects of PTIO and L-NNA are NO-independent.

Hyperpolarising effects of NO donors on the B2 neurons are mediated via extrasynaptic release of neuroactive substances from the buccal ganglia

The absence of hyperpolarising effects of NO donors on isolated B2 cells clearly indicated that these effects in situ are indirect and mediated via other neurons in the buccal ganglia. Notably, in situ, no typical inhibitory postsynaptic potentials, which could underlie this tonic influence, were observed. We hypothesised that extrasynaptic or volume neurotransmitter release may underlie these effects. To test this idea, we studied whether isolated B2 cells respond with a change in firing rate when placed back close to their initial position in the ganglion and whether the NO donors are capable of producing stronger hyperpolarising effects in these conditions. Earlier, this approach was used to detect extrasynaptic release from the pedal ganglia of *Lymnaea* (Chistopolsky and Sakharov 2003).

Isolated B2 cells placed close to their initial position (distance ca. 10% of cell diameter, Fig. 8a) in the buccal ganglia invariably changed the firing rate and/or membrane potential level ($n = 11$, Fig. 8b, c). In most cases, a depolarising influence of the ganglion was detected (delta membrane potential is $+8.2 \pm 1.4$ mV, the rate of firing: 3.8 ± 2 vs. 19.5 ± 8 far and near the ganglion, respectively, $n = 10$, $P = 0.02$, $z = 2.2$, paired Wilcoxon signed-rank test for dependent samples). This effect could be observed not only near the initial position of the B2 neuron, but also in the vicinity of some other parts of the ganglion surface ($n = 3$).

Addition of the NO donors (SNP and SNAP) to the flow, indeed, invariably resulted in the hyperpolarisation of isolated B2 neuron placed near its initial position in the buccal ganglion (Figs. 9b, c, 10). Delta membrane potential was -7 ± 2 mV, the rate of firing: 19.4 ± 8 versus 8 ± 4 far and near the ganglion, respectively, $n = 10$, $P = 0.02$, $z = 2.2$, paired Wilcoxon signed-rank test for dependent samples. Hyperpolarisation of isolated B2 neuron developed in parallel with activation of the buccal feeding rhythm (Fig. 10a). In 25% of cases, even changes of membrane potential similar to the giant hyperpolarising waves were observed in isolated B2 neurons (Fig. 9c), thus indicating a possible extrasynaptic origin of the giant hyperpolarising waves. This indicates that multiple extrasynaptic interactions might be involved in the communication within the buccal system, of which we can statistically confirm the NO-mediated hyperpolarisation of the B2 cell.

Discussion

In this work, we provide the first, to our knowledge, evidence that NO and extrasynaptic neurotransmitter release take part in the transient coordination of rhythm-generating units. Our data demonstrate that NO not only activates the buccal central pattern generator, as reported earlier (Moroz et al. 1993; Elphick et al. 1995), but in parallel also hyperpolarises the B2 bursters controlling gut contractions. The hyperpolarising effects of NO donors were absent in

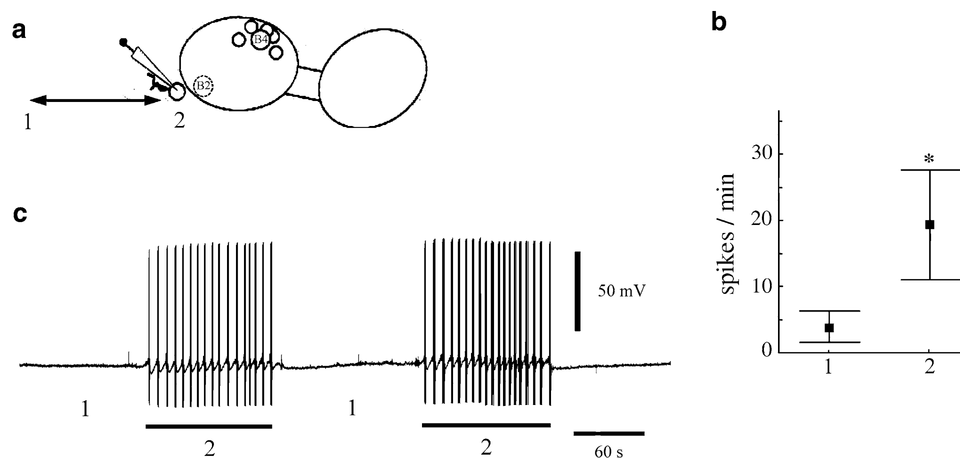

Fig. 8 Extrasynaptic influence of the buccal ganglia on the activity of the B2 neuron. **a** Schematic representation of the experiment (after Chistopolsky and Sakharov 2003). Isolated B2 cell impaled with the microelectrode was moved away from the ganglion at a distance of at least one ganglion diameter (*1* position) and placed back close to its initial position in the buccal ganglia (*2* position). **b** The mean firing rate of isolated B2 prior to (*1*) and after approach to the buccal ganglia (*2*); firing rate is measured in spikes/min, action potentials are measured per minute, and all values are reported as mean with SEM, $*P < 0.05$, the paired Wilcoxon test. **c** The response of isolated B2 neuron to the approach to its initial position in the buccal ganglia (*2*), horizontal line indicates B2 activity near the ganglion [*MP* −48 mV (**c**)]

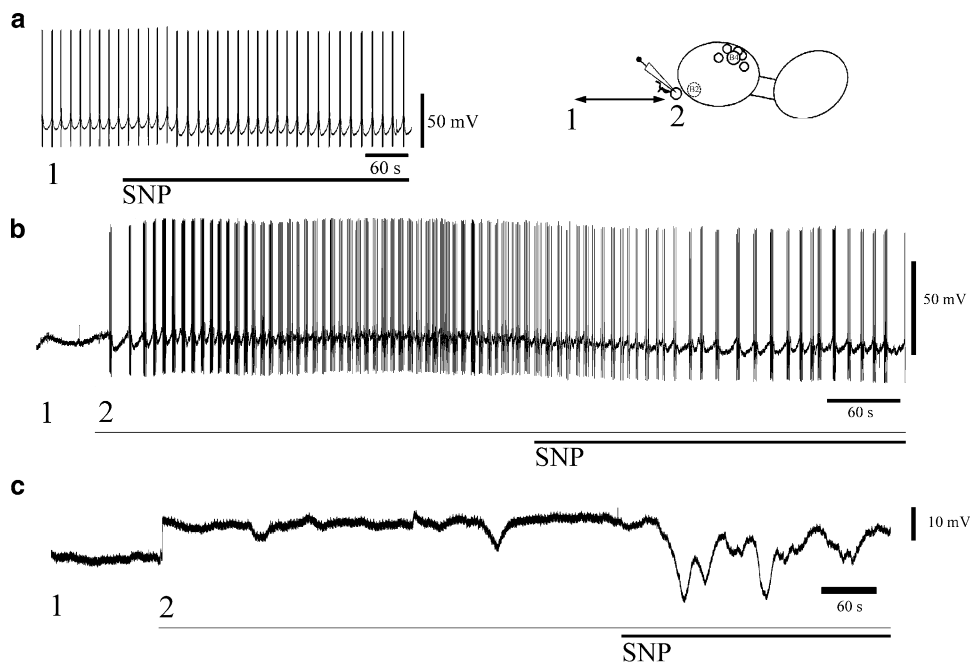

Fig. 9 Comparison of the effects of the NO donor sodium nitroprusside on isolated B2 neurons placed far and near to their initial position in the ganglion. **a** No effect of sodium nitroprusside (*SNP* 0.1 mM) on isolated B2 placed at a distance of at least one ganglion diameter (*1* position, the scheme in the right corner, see also statistics in Fig. 7a). **b**, **c** Hyperpolarising effect of *SNP*, 0.1 mM, on isolated B2 placed near the ganglion, position *2* [*MP* −48 mV (**b**), −65 mV (**c**)]. In **c**, extrasynaptic events similar to giant hyperpolarising waves can be observed in isolated B2 placed near the buccal ganglion caused by *SNP*. The *thin line* (in **b** and **c**) indicates that the B2 neuron is in the position *2* (near the ganglion), the *thicker horizontal line* with *SNP* abbreviation indicates the *SNP* presence in the Ringer stream as above. Extrasynaptic excitatory influence of the ganglion on the isolated B2 cell is also seen in **b** and **c**, note depolarisation and activation of the B2 after transition from *1* to *2* position

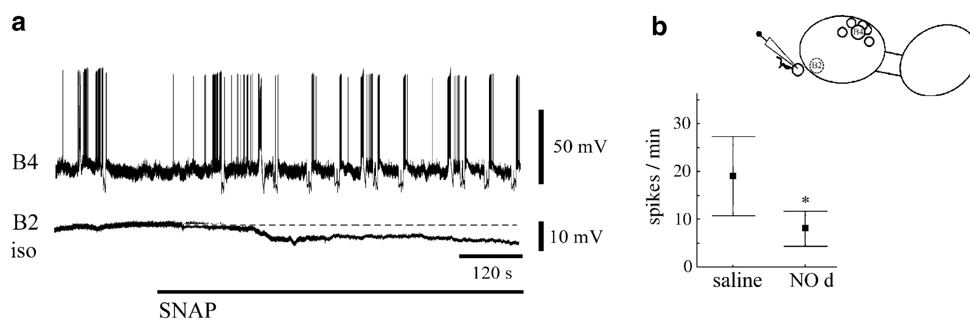

Fig. 10 NO donors are capable of affecting isolated B2 neurons placed near their initial position in the ganglion. **a** The simultaneous activity recording of the isolated B2 placed near the ganglion and the B4 neuron in the ganglion. *SNAP* 1 mM, activates buccal rhythm and simultaneously hyperpolarises the B2 neuron (the initial MP level is −43 mV). **b** The mean firing rate of isolated B2 near the ganglion surface prior to and in 5 min of treatment with NO donors (*SNP* and *SNAP*, pooled, firing rate is measured in spikes/min, action potentials are measured per minute, and all values are reported as mean with SEM, *$P < 0.05$, the paired Wilcoxon test)

isolated B2 cells as they moved away from the ganglion surface. However, in conditions excluding synaptic but permitting extrasynaptic chemical communication, i.e., when isolated B2 cells were placed close to their initial position in the buccal ganglia, NO was capable of causing hyperpolarisation in isolated B2 cells. This unambiguously suggests that extrasynaptic or volume communication is involved in the mechanism of coordination. Since the B2 neuron is a nitrergic cell, our findings can also be considered in terms of mechanisms of negative feedback regulation of the nitrergic system.

Reciprocal control of buccal central pattern generator and B2 bursters via NO

Both SNP and SNAP, NO donors tested in our experiments, activated buccal rhythm and hyperpolarised the B2

cells. Besides tonic hyperpolarisation, which was observed during the entire period of buccal rhythm generation, the NO donors also activated giant hyperpolarising waves associated with feeding cycles. The SNP effects were significantly decreased by an inhibitor of NO-sensitive guanylyl cyclase, thus indicating the involvement of guanylyl cyclase in the mechanism of these effects. The NO scavenger PTIO and the inhibitor of NO-synthase L-NNA depolarised the B2 cell, activated the B2 bursting and abolished the giant hyperpolarising waves. Taken together, these data suggest that the NO level may define the balance between buccal central pattern generator activity and B2 activity by having opposite influences on these two rhythm-generating units. Indeed, the level of membrane potential of the B2 neurons was significantly higher in untreated preparations with the active buccal central pattern generator than in untreated preparations with the silent pattern generator. This suggests that the B2 bursters are hyperpolarised during activation of buccal rhythm under natural conditions and confirms the existence of reciprocal regulation of the B2 neurons and central pattern generator for the radula rhythm. The role of NO in chemosensory activation of feeding behaviour was previously demonstrated in behavioural, electrophysiological and genetic experiments (Moroz et al. 1993, 1994; Elphick et al. 1995; Korneev et al. 2002). These data presumed a possible switch in cerebral ganglia from nitrergic to other chemical signal in the cerebral ganglia. In our experiments, the NO donors produced similar effects in isolated buccal ganglia, indicating that there are targets for NO in the buccal ganglia. Earlier, the conclusion that NO works not only as a modulator from sensory inputs, but also from a source within the buccal ganglia, was made from experiments on isolated B4 buccal motoneurons, which are sensitive to NO (Moroz et al. 2005). Earlier reported observations that giant hyperpolarising waves could be induced by stimulation of the B2 cell (Dyakonova and Dyakonova 2008b) clearly show that B2 neuron is one possible source of NO. The question of other possible sources of NO in the buccal ganglia is only partially resolved. Besides the B2 cell, numerous NADPh positive cell bodies and processes were previously observed in the buccal ganglia (Moroz et al. 1994; Sadamoto et al. 1998), but the presence of NO-synthase in these cells remains in question.

Figure 11 summarises the available data on relationships between NO, the feeding central pattern generator, and the nitrergic B2 bursters. It is evident that NO, including NO released from the B2 cells, activates the buccal central pattern generator and—indirectly, via volume neurotransmission of unknown origin—hyperpolarises the B2 neuron. The possible sources of volume release inhibiting the B2 bursters are presently unknown (marked with question mark on the scheme). They might be

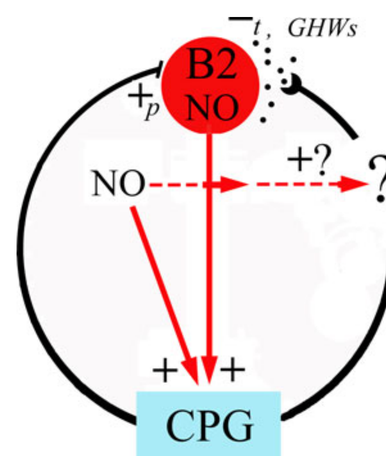

Fig. 11 Schematic representation of possible interactions between nitric oxide, the nitrergic B2 cells and buccal central pattern generator. Nitric oxide (NO), including NO released from the B2 cells, activates the buccal central pattern generator (*CPG*). NO indirectly, via volume neurotransmitter release of as yet unknown origin (marked with *question mark*), tonically hyperpolarises the B2 ($-t$) and induces giant hyperpolarising waves in the B2 (*GHWs*). Central pattern generator affects the activity of the B2 by earlier described phase-locked excitatory input ($+p$). Besides phasic excitation, activity of central pattern generator also results in tonic hyperpolarisation of the B2 and in occurence of giant hyperpolarising waves ($-t$, GHWs). Whether NO activates the cellular sources of volume inhibitory release directly or via buccal central pattern generator is unknown. Activation in response to NO is marked by *arrows* with + everywhere. Presumably synaptic, second-phase input to the B2 from central pattern generator is traditionally marked with *perpendicular line*. $+p$ indicates that this influence is phasic and excitatory. Volume transmission is schematically shown as varicose neurotransmitter release. $-t$ indicates that this influence is tonic and inhibitory

activated directly by NO or via activation of the buccal central pattern generator. Besides, tonic hyperpolarisation and giant hyperpolarising waves described here (right side of the scheme), there is also the excitatory phasic input to the B2 neurons from the generator (the left side), described earlier (Benjamin and Rose 1979; Park et al. 1998).

The B2 cell is an endogenous burster activated by decreases in the NO level

A fast-bursting rhythm was observed in the B2 cells earlier in situ (Benjamin and Rose 1979). Although it was suggested that this pattern was the cells' endogenous nature, the rhythm had not been seen in cultured isolated cells. In our experiments on freshly isolated cells, it was evident that the B2 is an endogenous burster that is capable of generating rhythmic bursts after complete isolation.

Results obtained earlier (Moroz et al. 2005) as well as those presented here indicate that NO donors do not have any significant effect on the electrical activity of either

cultured or freshly isolated B2 neurons. Surprisingly, we observed that the excitatory effects of the NO synthase inhibitor or the NO scavenger persisted after complete isolation of the B2 neuron. Moreover, these drugs were sometimes potent enough to activate the bursting rhythm in the initially silent isolated B2 cells. Strikingly, this indicates that B2 cells may possess an endogenous mechanism of activation in response to NO deficiency. ODQ produced weaker polarising effects. PTIO had an additional effect in the presence of ODQ. This suggests either that there are other targets for NO in the B2 cells besides cGMP cyclase, for example, proteins that can be directly nitrozilated by NO, or, alternatively, that some of the depolarising effects of PTIO and L-NNA are NO-independent. Indeed, both drugs are potentially capable of affecting metabolic events, for example, L-NNA might interfere with mitochondrial function (Addabbo et al. 2009), while PTIO might scavenge O_2 and other gaseous substances (Goldstein et al. 2003). Non-specific action of ODQ neither can be excluded.

Extrasynaptic neurotransmitter release is involved in transient coordination of buccal central pattern generator and B2 bursters

The theory of non-synaptic chemical communication has been developed since the early 1990s (Sakharov 1990; Agnati et al. 1995, 2006). There is now growing evidence that extrasynaptic neurotransmitter release does play an important role in interneuronal communication. A significant percentage of receptors are localised extrasynaptically. Real-time extrasynaptic release of serotonin, dopamine, noradrenaline, glutamate and GABA was detected using amperometry in various parts of the mammalian brain (Bunin and Wightman 1998; Jaffe et al. 1998; Vizi et al. 2004; Sem'yanov 2005; Chen et al. 2008). In invertebrates of various taxa, such as leeches, insects, nematodes and molluscs, extrasynaptic neurotransmitter release has also been clearly detected (Bruns and Jahn 1995; Chen et al. 1995; Spencer et al. 2000; De-Miguel and Trueta 2005). Recently, extrasynaptic release of serotonin and other substances from the pedal ganglia of *Lymnaea stagnalis* was shown by pharmacological experiments and the use of biosensors in our laboratory (Chistopolsky and Sakharov 2003; Dyakonova et al. 2009).

Here, we demonstrate for the first time that extrasynaptic release may be involved in transient reciprocal coordination of rhythm generating units, namely, the central pattern generator for the radula rhythm and B2 bursters controlling gut motility. Hyperpolarising effects of NO donors on the B2 neurons were observed either in situ or when isolated B2 cells were placed near their initial position in the ganglion, but not in isolated B2 cells moved away from the ganglia. These results clearly suggest that the hyperpolarising effects of NO depend upon extrasynaptic release from the buccal ganglia. Theoretically, two possible mechanisms may mediate this phenomenon: (1) extrasynaptic release changes the B2 properties so that the cell becomes sensitive to NO, or (2) NO donors change extrasynaptic release from the ganglia and thus indirectly affect the B2 neurons. The second mechanism seems to be more probable; however, unambiguous discrimination requires additional study. In both cases, however, volume extrasynaptic interactions between the B2 neurons and the buccal ganglia are required.

Tonic extrasynaptic influence is likely to also explain the difference in the firing rate of B2 neurons in situ and after isolation. The change in the firing rate of the B2 neurons in response to the approach of the buccal ganglia clearly shows that, as in the case of the pedal ganglia (Chistopolsky and Sakharov 2003), there is tonic extrasynaptic release from the buccal system. The release is likely to be capable of affecting many other cells in the ganglion, and it is therefore possible that *Lymnaea's* feeding central pattern generator in general, one of the best studied model systems, is under the influence of extrasynaptic release. Similar non-synaptic interactions were found earlier in swimming central pattern generator of *Clione limacine* (Arshavsky et al. 1988). All these data indicate that extrasynaptic influence must play a significant role and is presently not sufficiently understood.

The intensity of extrasynaptic neurotransmitter release was previously shown to be affected by changes in the membrane potential of a neuron by electrical stimulation (De-Miguel and Trueta 2005) and by changes in the intensity of the synthesis of neurotransmitters (Dyakonova et al. 2009). As far as we know, the results presented here are the first indication that NO may significantly affect extrasynaptic neurotransmitter release. Whether the effect is due to NO-induced depolarisation of intermediate neurons or to direct NO influence on the proteins associated with neurotransmitter release or uptake (Jansson et al. 1999; Kiss et al. 2004) remains to be elucidated.

To conclude, the results presented here suggest that (1) NO may be involved in coordination between the radula and foregut movements via reciprocal influences on the buccal central pattern generator and the B2 neuron; (2) extrasynaptic neurotransmitter release takes part in transient coordination of rhythm-generating units as well as in negative feedback regulation of the nitrergic system.

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