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Excitatory actions of GABA mediate severe-hypoxia-induced depression of neuronal activity in the pond snail (*Lymnaea stagnalis*)

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Summary

To characterize the effect of severe hypoxia on neuronal activity, long-term intracellular recordings were made from neurones in the isolated central ring ganglia of *Lymnaea stagnalis*. When a neurone at rest in normoxia was subjected to severe hypoxia, action potential firing frequency decreased by 38% (from 2.4–1.5 spikes s^{-1}), and the resting membrane potential hyperpolarized from –70.3 to –75.1 mV. Blocking $GABA_A$ receptor-mediated synaptic transmission with the antagonist bicuculline methiodide (100 μmol l^{-1}) decreased neuronal activity by 36%, and prevented any further changes in response to severe hypoxia, indicating that GABAergic neurotransmission mediates the severe hypoxia-induced decrease in neuronal activity. Puffing 100 μmol l^{-1} GABA onto the cell body produced an excitatory response characterized by a transient increase in action potential (AP) firing, which was significantly decreased in severe hypoxia. Perturbing intracellular chloride concentrations with the $Na^+/K^+/Cl^-$ (NKCC1) cotransporter antagonist bumetanide (100 μmol l^{-1}) decreased AP firing by 40%, consistent with GABA being an excitatory neurotransmitter in the adult *Lymnaea* CNS. Taken together, these studies indicate that severe hypoxia reduces the activity of NKCC1, leading to a reduction in excitatory GABAergic transmission, which results in a hyperpolarization of the resting membrane potential (V_m) and as a result decreased AP frequency.

Key words: GABA, *Lymnaea stagnalis*, hypoxia.

Introduction

The cellular and physiological responses to a near lack of oxygen differ from those during more routine moderate hypoxia. For example, anoxia rapidly depresses neuronal activity and synaptic transmission in rat (Haddad and Jiang, 1997; Pena and Ramirez, 2005; Zhu and Krnjevic, 1994) and turtle brain (Feng et al., 1988; Lutz, 1992). These severe-hypoxia-induced decreases in neuronal activity and synaptic transmission are not surprising given the high metabolic cost of these processes (Attwell and Laughlin, 2001; Seisjo, 1978). For example, a detailed energy expenditure budget of grey matter in the rodent brain reveals that action potential generation and propagation consumes 47% of available ATP, while postsynaptic potentials consume an additional 34% (Attwell and Laughlin, 2001). GABAergic (γ-aminobutyric acid) neurones make widespread connections within neuronal networks and thus are capable of controlling network oscillations and patterns of activity in numerous systems and organisms. GABA modulates oscillatory networks in the olfactory system of the terrestrial slug *Limax marginatus* (Bazhenov et al., 2001; Ito et al., 2004), as well as suppressing the oscillatory activity of crustacean pyloric neurones (Cazalets et al., 1987). Thus GABAergic transmission in *Lymnaea* is well-positioned to regulate severe-hypoxia-induced decreases in neuronal activity that are required to preserve limited ATP stores.

GABAergic synaptic transmission itself is modulated by decreases in oxygen; extracellular levels of GABA rise in the anoxic brains of the shore crab *Carcinus maenas* (Nilsson and Winberg, 1993), Crucian carp (Hylland and Nilsson, 1999) and turtle *Trachemys scripta* (Nilsson and Lutz, 1991). The ability of oxygen to modulate GABAergic transmission, coupled with the ability of GABAergic transmission to regulate neuronal network activity, led to our hypothesis that modulation of GABAergic neurotransmission may be responsible for severe-hypoxia-induced decreases in neuronal activity.

To test this hypothesis we took advantage of the anoxia-tolerant pulmonate pond snail *Lymnaea stagnalis,* which survives about 40 h in a N_2-bubbled environment at 20°C (Wijsman et al., 1985). It has also been used extensively for studies on respiratory neurophysiology, mainly because of its well characterized respiratory system and behaviours (Inoue et al., 2001; Syed et al., 1990; Taylor and Lukowiak, 2000). In this study, we demonstrate that under normoxic conditions GABA acts as an excitatory neurotransmitter in neurones of the dorsal pedal ganglia, and that during severe hypoxia the excitatory actions of GABA are significantly decreased. The

severe-hypoxia-induced decrease in excitatory GABAergic transmission occurs *via* a decrease in cation–chloride cotransporter (NKCC1) activity, and accounts for the severe-hypoxia-induced decrease in action potential firing and hyperpolarization of the resting membrane potential.

Materials and methods

Animals

Laboratory-raised stocks of the freshwater snail *Lymnaea stagnalis* L were maintained in aquaria filled with well-aerated filtered water at 22°C. *Lymnaea* were fed organic lettuce leaves and carrot shavings every 2 days. Experiments were performed on snails 20–30 mm in length (~3 months old).

Solutions

Lymnaea saline solution included (in mmol l^{-1}): 10 glucose; 51.3 NaCl; 1.7 KCl; 4 $CaCl_2$; 1.5 $MgCl_2$; 10 Hepes; pH 7.9 using NaOH; 144 mOsm. The extracellular chloride concentration in this saline solution was based on previous measurements of *Lymnaea* haemolymph (de With and van der Schors, 1984). Normoxic *Lymnaea* saline solution was prepared by bubbling air directly into the solution for a minimum of 30 min. Severely hypoxic *Lymnaea* saline solution was prepared in an identical manner with 100% N_2 replacing air. Perfusion tubes for the hypoxic saline were double jacketed, and the outer jacket gassed with 100% N_2. A Clark-type oxygen electrode and computer based acquisition software (Qubit System, Kingston, Ontario, Canada) was used to measure the partial pressure of oxygen (P_{O_2}) in the perfusion chamber. Following perfusion with anoxic saline, P_{O_2} in the recording chamber decreased from approximately 155 mmHg (1 mmHg=133.3 Pa) P_{O_2} (normoxia) to 7 Pa P_{O_2} (severe hypoxia) in less than 10 min. The recording chamber was open to room air making anoxia difficult to achieve.

Isolated brain preparations

Snails were anesthetized briefly in *Lymnaea* saline solution containing 30% Listerine, a standard anaesthetic used in *Lymnaea* studies (Spencer et al., 2002), then de-shelled with forceps. *Lymnaea* were pinned dorsal surface up to the bottom of a Sylgard-filled dissection dish, covered with *Lymnaea* saline solution, and a medial incision was made from the base of the mantle to the head. The central ring ganglia were removed with a pair of fine surgical scissors, and transferred immediately to a recording dish with 3 ml of fresh *Lymnaea* saline solution, and pinned out (Fig. 1A). The connective tissue sheath was removed from the left and right pedal ganglia (LPeDG and RPeDG, respectively) with a pair of fine forceps, prior to being immediately transferred to the microscope stage where they were perfused with normoxic *Lymnaea* saline solution at a rate of 3 ml m^{-1} at 22°C.

Electrophysiology

Intracellular recordings were made from neurones on the dorsal surface of either the L- or RPeDG (Fig. 1A,B) visualized with IR-DIC optics using an Olympus BX51WI microscope. Specifically, using the morphological and electrophysiological map of the *Lymnaea* pedal ganglia, recordings were restricted to non-identified neurones of neuronal cluster F (Fig. 1B) (Kyriakides et al., 1989). Glass microelectrodes (#50812 Stoeling Co., Wood Dale, IL, USA) were pulled with a P97 micropipette puller (Suttert Instruments, Novato, CA, USA) and filled with a saturated solution of K_2SO_4 (tip resistance 50–60 MΩ). Visualized neurones were impaled using a Siskiyou Micromanipulator (#MX7600; Grants Pass, Oregon, USA). The intracellular signals were amplified with an Axoclamp 2B and recorded using Clampex 7 software (Molecular Devices; Union City, CA, USA). Electrophysiological experiments were analyzed using Clampfit 9.1 software (Molecular Devices; Union City, CA, USA).

Impaled neurones were maintained under normoxic conditions until action potential (AP) frequency and resting membrane potential (V_m) stabilized (~10 min). AP frequency and V_m were then recorded for a minimum of 20 min under normoxic conditions. Following a 10 min switch from normoxic to hypoxic saline, AP frequency and V_m were again recorded for another 20 min period. Following this hypoxic recording, neurones were returned to normoxia for the duration of the recording. AP frequency was determined by analyzing V_m for 8.8 s every 20 s, at a sampling rate of 5 kHz. There was significant variation in the AP frequency between neurones (minimum number of APs in 8.8 s: 2.72±0.24 or 0.31 spikes s^{-1}; maximum number of APs in 8.8 s: 47.12±2.05 or 5.35 spikes s^{-1}). Therefore, in order to compare AP frequency between neurones, data were normalized to a control period.

Chemicals

γ-Aminobutyric acid (GABA; A5838, Sigma, Oakville, Ontario, Canada) was dissolved in *Lymnaea* saline solution and applied to the pedal ganglia using a VC-6 perfusion valve control system (Warner Instruments, Handen, CT, USA) at a final concentration of 500 μmol l^{-1}. A 100 mmol l^{-1} stock solution of bicuculline methiodide (B6889, Sigma) was prepared in dimethyl sulfoxide (DMSO; D5879, Sigma); the stock was diluted to a final concentration of 100 μmol l^{-1} in either normoxic or hypoxic *Lymnaea* saline solution. A 100 mmol l^{-1} stock solution of bumetanide (B3023, Sigma) was prepared in ethanol and diluted to a final concentration of 100 μmol l^{-1} in normoxic saline solution.

Statistical analysis

All averages are reported as means ± standard error (s.e.m.). Statistical analysis was performed using SigmaStat software (Point Richmond, CA, USA).

Results

The effects of severe hypoxia on neuronal activity were determined by monitoring AP firing frequency and V_m. Intracellular recordings were obtained from visualized

neurones in cluster F (with the exception of identified neurones L- and RPeD1) on the dorsal surface of the left or right pedal ganglion (L- or RPeDG) of the central ring ganglia isolated from *L. stagnalis,* as shown in Fig. 1A,B. Under control normoxic conditions there were no significant changes in AP frequency for over 100 min of recording [normoxia (first half of the recording) → normoxia (second half of the recording) = 1.9±0.6→1.8±0.6 spikes s^{-1}; *N*=9, *P*=0.935 paired *t*-test; Fig. 2A]. Switching from normoxic saline to a severely hypoxic saline solution (100% N_2 bubbled) induced a sustained 38% decrease in AP frequency (normoxia → hypoxia = 2.4±0.7→1.5±0.6 spikes s^{-1}; *N*=9, *P*=0.001 paired *t*-test; Fig. 2A,B). After a 40 min exposure to severe hypoxia, normoxic conditions were resumed, but no restoration to normoxia AP frequency was observed (in the 1 h following the return from hypoxia to normoxia).

V_m was also determined under both normoxic and hypoxic conditions (Table 1). When neurones were kept entirely normoxic there was no significant difference between V_m in the first and second halves of the recordings (–69.3±2.9→ –69.7±2.9; *N*=10, *P*=0.92 paired *t*-test). However, under hypoxic conditions V_m is significantly hyperpolarized, as compared with normoxia (–70.3±3.0→–75.1±4.5; *N*=14, *P*=0.001 paired *t*-test). Thus, neurones in the isolated central ring ganglia of *Lymnaea* respond to severe decreases in oxygen with a significantly reduced AP frequency and hyperpolarized V_m.

All neurones were initially spontaneously active at their resting membrane potential, showing APs, and excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs; Fig. 3A). Inhibitory neurotransmission regulates the overall level of network activity in numerous preparations (Whittington and Traub, 2003), and so we hypothesized that the hypoxia-induced decrease in AP frequency may result from a modulation of inhibitory neurotransmission. Because classic fast inhibitory neurotransmission is commonly mediated by GABA binding to a Cl^- permeant $GABA_A$ receptor, we examined the role of $GABA_A$-mediated neurotransmission in the hypoxia-induced decrease of neuronal activity. We repeated the normoxia → hypoxia experiments in the presence of the $GABA_A$ receptor antagonist bicuculline methiodide (100 μmol l^{-1}). The addition of bicuculline to the perfusate induced a 36% decrease in AP frequency from neurones resting in normoxia (Fig. 3B; normoxia → normoxia + bicuculline= 3.3±0.6→2.1±0.5 spikes s^{-1}, *N*=7, *P*=0.003 paired *t*-test). The decrease in AP frequency observed when $GABA_A$ neurotransmission was blocked with bicuculline was not significantly different from the decrease in AP frequency elicited by severe hypoxia (Fig. 3B; *P*=0.84). Normoxic neurones exposed to bicuculline showed no further depression in AP frequency upon switching the perfusate to hypoxia + bicuculline (Fig. 3B; normoxia + bicuculline → hypoxia + bicuculline=2.5±1.2→1.9±0.9 spikes s^{-1}, *N*=6, *P*=0.235 paired *t*-test). Likewise, blocking $GABA_A$ neurotransmission prevented the V_m hyperpolarization that occurred during the antagonist-free normoxia → hypoxia experiment (Table 1; V_m normoxia + bicuculline=–72.3±1.9, V_m hypoxia + bicuculline=–72.6±1.9; *N*=6, *P*=0.8 paired *t*-test). Thus GABAergic synaptic transmission appears to be required for both the hypoxia-induced decrease in neural activity and V_m hyperpolarization.

Neurones in the mature mammalian CNS maintain a low level of intracellular Cl^- ($[Cl^-]_i$), thus when GABA binds to a Cl^- permeant $GABA_A$ receptor it hyperpolarizes the postsynaptic membrane. However, in the immature mammalian CNS and in certain brain structures of the mature CNS, intracellular levels of Cl^- are high ($[Cl^-]_i>[Cl^-]_e$) rendering GABAergic transmission depolarizing and excitatory (Ben-Ari, 2002). The observed hypoxia-induced decrease in AP frequency could be explained by either: (1) an increase in hyperpolarizing GABAergic transmission; or (2) a decrease in depolarizing GABAergic neurotransmission. In normoxia, the application of 500 μmol l^{-1} GABA produced an acute 63% increase in AP firing and a 17±1 mV depolarization of V_m

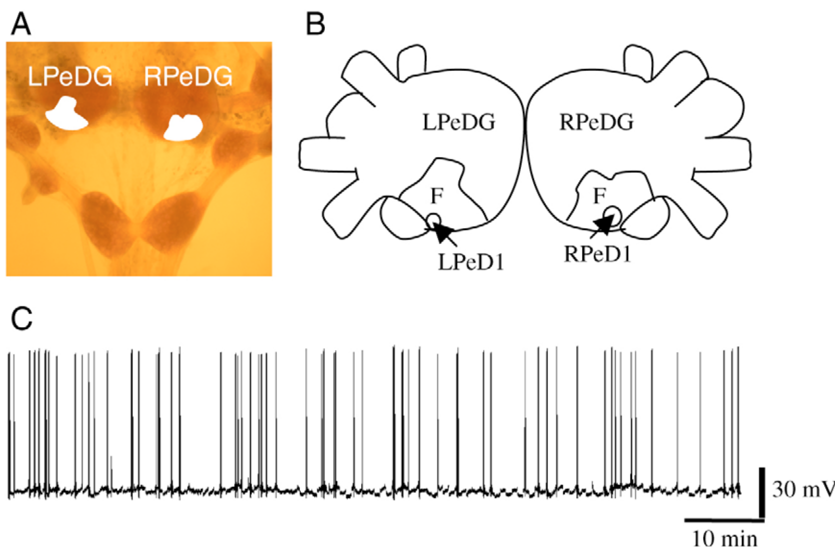

Fig. 1. Electrophysiological recording from neurones of the *Lymnaea* pedal ganglia. (A) The central ring ganglia isolated from *Lymnaea*. Intracellular recordings were made from neurones in cluster F (white region) on the dorsal surface of either the left or right pedal ganglion (LPeDG or RPeDG). (B) Schematic of the *Lymnaea* central ring ganglia. Recordings were not obtained from the identified neurones L- or RPeD1. (C) An example trace of a recording from a cluster F neurone maintained entirely under normoxic conditions.

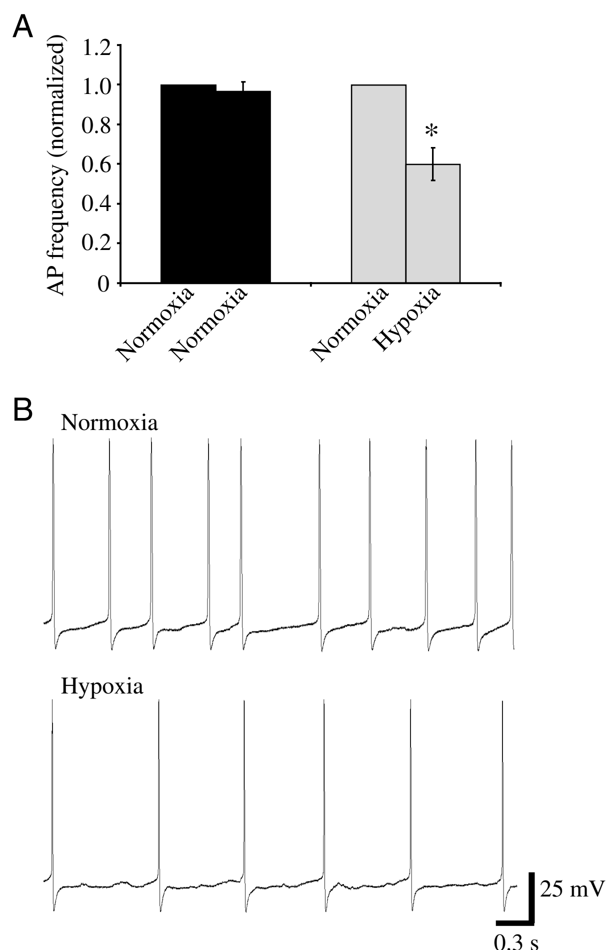

Fig. 2. Severe hypoxia decreases neuronal activity. (A) No significant changes in the action potential (AP) frequency were observed in normoxic neurones throughout the entire recording period (black bars; N=9, P=0.935 paired t-test). After switching from normoxia to severe hypoxia, AP frequency decreased significantly (grey bars; N=9, P=0.001 paired t-test). (B) Example traces of AP frequency during normoxia and 10 min after the switch to severe hypoxia.

(N=10; Fig. 4B), demonstrating that GABA is acting as an excitatory neurotransmitter in these neurones. Severe hypoxia produced statistically significant decreases in this excitatory response (Fig. 4B; N=7, P=0.037 paired t-test); the AP firing in response to GABA decreased to 33% while the GABA-induced depolarization decreased to 6±2 mV. Thus, severe hypoxia significantly decreases the magnitude of excitatory GABAergic neurotransmission in *Lymnaea*.

As described earlier, the polarity of GABAergic neurotransmission is dependent upon the concentration of $[Cl^-]_i$, which in neurones is mainly determined by the differential expression of two cation-chloride cotransporters (Payne et al., 2003): NKCC1 ($Na^+/K^+/2Cl^-$), a cotransporter that actively accumulates Cl^- into the cell (Delpire, 2000); and the neurone-specific KCC2 (K^+/Cl^-), which extrudes Cl^-. To examine if the cotransporter NKCC1 was largely responsible for maintaining relatively high $[Cl^-]_i$ rendering GABA excitatory in our preparation, we observed the effect of the NKCC1 antagonist bumetanide (100 μmol l^{-1}) on AP frequency and V_m. When 100 μmol l^{-1} bumetanide was added to the bath, neurones resting in normoxic solution showed a significant decrease in their AP frequency (Fig. 5A,B; normoxia → normoxia + bumetanide=3±0.8→1.8±0.5 spikes s^{-1}, N=6, P=0.01 paired t-test). In three out of 15 recordings, APs were absent following bumetanide or hypoxia, such as in Fig. 5B. This decrease was not significantly different from the decrease observed when neurones underwent the normoxia → hypoxia switch. The significant decrease in AP frequency induced by bumetanide is readily observed in Fig. 5B, which shows an abolishment of APs. When bumetanide blocks the inward movement of Cl^-, $[Cl^-]_i$ decreases resulting in less excitatory GABAergic transmission, causing a hyperpolarizing of V_m (Table 1; V_m normoxia=–64.5±3.63, V_m normoxia + bumetanide=–72.3±1.9; N=6, P=0.03 paired t-test), thus decreasing AP frequency.

Discussion

In this study, we investigated the effects of severe hypoxia on neuronal activity, using the isolated central ring ganglia preparation from the pulmonate pond snail *Lymnaea stagnalis*. We observed that severe hypoxia hyperpolarized the resting membrane potential and decreased action potential firing by 38%. When $GABA_A$-receptor mediated neurotransmission was antagonized, severe hypoxia had no effect on neuronal activity. By puffing GABA onto the cell body, and perturbing

Table 1. *Effects of hypoxia and modulated inhibitory neurotransmission on membrane potential*

No. of cells	Membrane potential V_m (V)						Statistical significance
	Normoxia	Normoxia	Hypoxia	Normoxia+ bicuculline	Hypoxia+ bicuculline	Normoxia+ bumetanide	
10	–69.3±2.9	–69.7±2.9					No; P=0.92
14	–70.3±3.0		–75.1±4.5				Yes; P=0.001
6	–65.8±3.6			–71.4±3.3			Yes; P=0.02
6				–72.3±1.9	–72.6±1.9		No; P=0.8
6	–64.5±3.6					–72.3±4.7	Yes; P=0.03

Values are means ± s.e.m. Statistical significance was determined using paired t-tests.

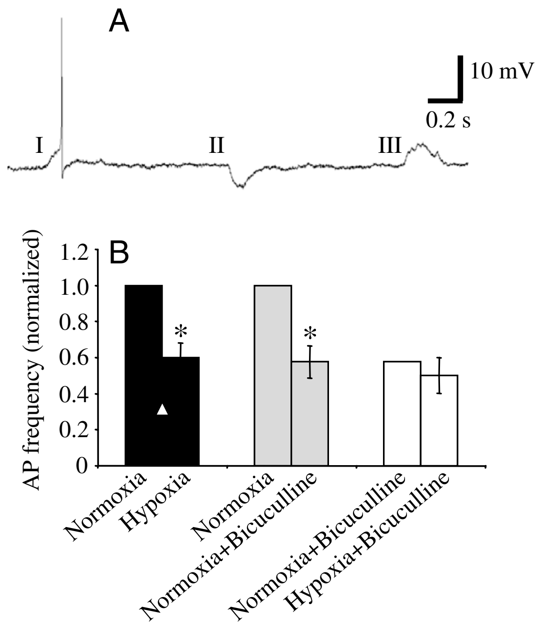

Fig. 3. $GABA_A$ receptors mediate hypoxia-induced neuronal depression. (A) When neurones were recorded at rest under normoxic conditions action potentials (APs) (at I), inhibitory postsynaptic potential (IPSPs; at II) and excitatory postsynaptic potential (EPSPs; at III) were observed. (B) Addition of $100\,\mu mol\,l^{-1}$ bicuculline to the perfusate induced a significant decrease in AP frequency (grey bars; N=7, P=0.003 paired t-test). When neurones underwent a normoxic to hypoxic transition in the presence of $100\,\mu mol\,l^{-1}$ bicuculline, there was no significant change in AP frequency (white bars; N=6, P=0.235 paired t-test). The hypoxia-mediated decrease in AP frequency is not significantly different from the decrease observed during the normoxic application of bicuculline (black hypoxia bars *vs* grey normoxia+bicuculline bars; P=0.844 unpaired t-test). Data sets denoted by a white triangle were taken from Fig. 2A for direct visual comparison.

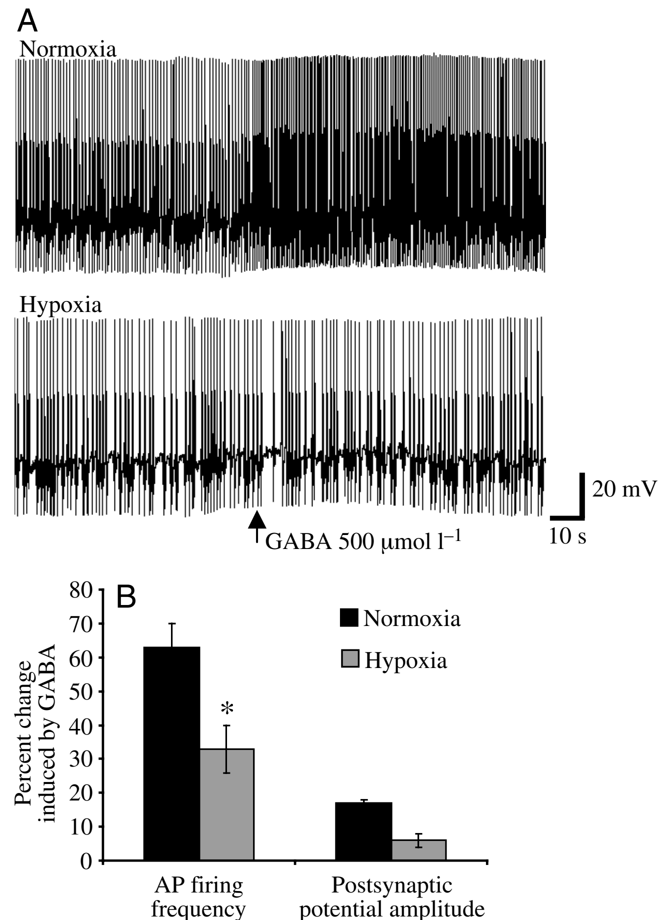

Fig. 4. Severe hypoxia decreases excitatory GABAergic neurotransmission in the *Lymnaea* central ring ganglia. (A) Example traces demonstrating the change in action potential (AP) firing frequency produced by puffing $500\,\mu mol\,l^{-1}$ GABA (at arrow) onto the central ring ganglia. In normoxia (top trace) GABA produced an increase in AP firing frequency. Switching from normoxia to hypoxia decreased the excitatory response to GABA (lower trace). (B) Bar graph summarizing the percentage change in AP firing frequency and postsynaptic potential amplitude induced by puffing $500\,\mu mol\,l^{-1}$ GABA onto both normoxic and hypoxic central ring ganglia.

intracellular chloride concentrations, we further demonstrate that GABA acts as an excitatory neurotransmitter in the *Lymnaea* CNS. Thus, during normoxia, excitatory GABAergic neurotransmission appears to maintain neuronal activity, such that the neurones are spontaneously active at rest. The severe hypoxia-induced decrease in excitatory GABAergic neurotransmission may act *via* regulation of NKCC1 to hyperpolarize the resting membrane potential and decrease spontaneous action potential firing. The severe hypoxia-induced decrease in neuronal activity would promote survival by significantly reducing the brain's energetic requirements in the face of limited ATP stores.

Experiments were restricted to neurones in cluster F of the dorsal pedal ganglia in order to take advantage of their similar electrophysiological properties (Kyriakides et al., 1989) and proximity to GABAergic neurones (Hatakeyama and Ito, 2000). The dorsal pedal ganglia each have three pairs of GABA-like immunoreactive cell clusters that make widespread connections in the CNS of adult *Lymnaea stagnalis.* GABA binds to the *Lymnaea* $GABA_A$ receptor, a multi-subunit membrane-spanning complex containing an integral chloride ion channel, which shares 30–50% similarity with the vertebrate $GABA_A$ receptor (Barnard et al., 1988; Harvey et al., 1991). The excitatory actions of GABA in this study are consistent with those previously reported, where GABA evoked a depolarization of the membrane potential on the majority of *Lymnaea* neurones studied (Rubakhin et al., 1996). In fact, GABA acts as both an excitatory and inhibitory neurotransmitter in the CNS of several gastropods (Alkon et al., 1992; Kim and Takeuchi, 1990; Yarowsky and Carpenter, 1977; Zhang et al., 1997). It will be interesting to know whether hypoxia has a similar depressive effect on excitatory GABA inputs in other organisms that are routinely challenged by hypoxia.

One also has to consider the possibility that in our study

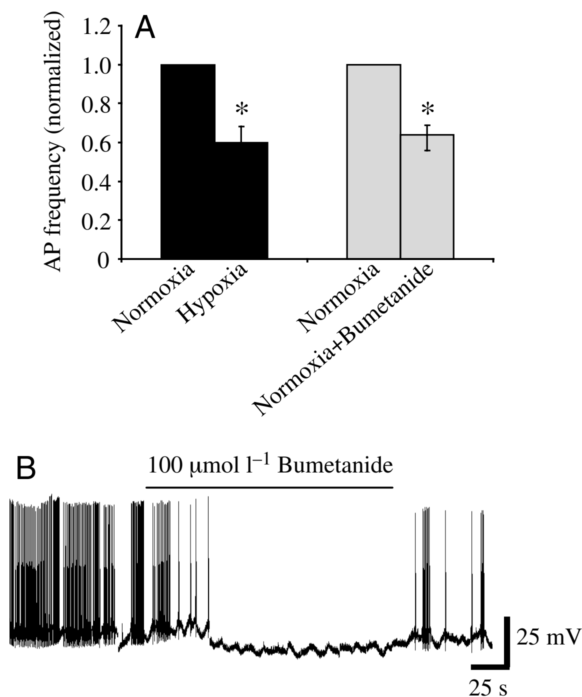

Fig. 5. NKCC1 renders GABA excitatory in the *Lymnaea* CNS. (A) Addition of the NKCC1 antagonist bumetanide (100 μmol l^{-1}) to the perfusate significantly decreases action potential (AP) firing frequency (grey bars; N=6, P=0.01 paired t-test). The normoxic decrease in AP frequency caused by bumetanide is not significantly different from the decrease observed in the normoxia to hypoxia switch (black bars *vs* grey bars; P=0.844 unpaired t-test). (B) Spontaneous firing of APs was prevented by the addition of 100 μmol l^{-1} bumetanide (at bar).

GABA may also be activating the K^+-permeable $GABA_B$ receptor. In fact, GABA is known to exert excitatory effects through $GABA_B$Rs in the *Lymnaea* osphradium (Kamardin et al., 1999). However, the involvement of excitatory $GABA_B$ in our study is unlikely based on the fact that the $GABA_A$R antagonist bicuculline entirely abolished the hypoxia-induced decrease in neuronal activity.

The present results are consistent with those previous published by Inoue et al. (Inoue et al., 2001) who demonstrated that perfusion with anoxic saline decreases the action potential firing frequency of respiratory pattern-generating neurones in *Lymnaea* (Inoue et al., 2001). Inoue et al. also report an inability to rescue baseline neuronal activity (Inoue et al., 2001). In our study, the inability to rescue was not due to a glycolytic substrate limitation, as glucose was included in the extracellular recording solution. It is also important to note that the decrease in AP activity and hyperpolarization of V_m did not appear to adversely affect neuronal health, as APs were still present. Interestingly, the hypoxia-induced changes in GABA neurotransmission reported in turtle CNS, in particular the rise in $GABA_A$ receptor number, are maintained for at least 24 h (Lutz and Leone-Kabler, 1995). Thus, the inability of normoxia to rescue the membrane potential and spontaneous action potential firing probably results from the activation of mechanisms that are maintained significantly longer than the hypoxic exposure.

Neuronal intracellular chloride is regulated by the opposing actions of two electroneutral cation–chloride cotransporters: NKCC1 and KCC2. Under physiological conditions NKCC1 transports $1Na^+:1K^+:2Cl^-$ into cells, while KCC2 transports $1K^+:1Cl^-$ out. The differential expression of these two transporters is what sets the reversal potential for Cl^- in neurones: when KCC2 dominates $[Cl^-]_i$ is low and GABA is inhibitory, when NKCC1 is dominant $[Cl^-]_i$ is high and GABA is excitatory. Based on the excitatory effects of GABA in these neurones we examined the effects of antagonizing NKCC1 with bumetanide and found a significant decrease in AP firing. This result is consistent with the previous observation that bumetanide rapidly inhibited the spontaneous bursts of individual pyramidal cells from rat hippocampus (Sipila et al., 2006).

Based on the results from the present study we suggest the following mechanism to account for how severe hypoxia depresses neuronal activity: in normoxia NKCC1 maintains high $[Cl^-]_i$, which renders GABA an excitatory neurotransmitter, since Cl^- would excite the cell when the $GABA_A$ receptor is activated. Severe hypoxia reduces the activity of NKCC1, which results in a decrease in $[Cl^-]_i$, which in turn decreases the magnitude of the excitatory GABAergic neurotransmission; decreasing excitation through $GABA_A$ receptors would decrease the excitatory drive to the recorded neurones resulting in a hyperpolarization of the resting V_m and, as a result, decreased AP frequency. This proposed mechanism can be tested in the future by: (1) examining the response of isolated *Lymnaea* neurones in cell culture to severe hypoxia; and (2) demonstrating directly the relationship between GABAergic neurotransmission and NKCC1 regulation of $[Cl^-]_i$.

List of abbreviations

GABA	γ-amino butyric acid
ATP	adenosine triphosphate
P_{O_2}	partial pressure of oxygen
mOsm	milliOsmol
PeDG	pedal ganglia
AP	action potential
V_m	membrane potential
EPSP and IPSP	excitatory and inhibitory synaptic potential

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