Comparative Biochemistry and Physiology, Part C xxx (2008) xxx-xxx

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Evidence of anoxia-induced channel arrest in the brain of the goldfish (*Carassius auratus*)☆

Michael P. Wilkie [a,*], Matthew E. Pamenter [b], Samir Alkabie [a], Dejana Carapic [a], Damian S.H. Shin [b], Leslie T. Buck [b]

[a] Department of Biology, Wilfrid Laurier University, 75 University Avenue West, Waterloo, Ontario, Canada N2L 3C5
[b] Department of Cell and Systems Biology and Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario, Canada M5A 2G6

ARTICLE INFO

Article history:
Received 6 March 2008
Received in revised form 15 June 2008
Accepted 15 June 2008
Available online xxxx

Keywords:
NMDA receptor
Na^+/K^+-ATPase
MK801
Ethanol
Oxygen
Gills

ABSTRACT

The common goldfish (*Carassius auratus*) is extremely anoxia tolerant and here we provide evidence that "channel arrest" in the brain of these fish contributes to ATP conservation during periods of anoxia. Whole-cell patch-clamp recordings of slices taken from the telencephalon indicated that the *N*-methyl-D-aspartate (NMDA) receptor, an ionotropic glutamate receptor and Ca^{2+}-channel, underwent a 40–50% reduction in activity during 40 min of acute anoxia. This is the first direct evidence of channel arrest in an anoxia-tolerant fish. Because goldfish produce ethanol as a byproduct of anaerobic metabolism we then conducted experiments to determine if the observed reduction in NMDA receptor current amplitude was due to inhibition by ethanol. NMDA receptor currents were not inhibited by ethanol (10 mmol L^{-1}), suggesting that channel arrest of the receptor involved other mechanisms. Longer-term (48 h) *in vivo* exposure of goldfish to anoxic conditions (less than 1% dissolved O_2) provided indirect evidence that a reduction in Na^+/K^+-ATPase activity also contributed to ATP conservation in the brain but not the gills. Anoxia under these conditions was characterized by a decrease in brain Na^+/K^+-ATPase activity of 30–40% by 24 h. Despite 90% reductions in the rates of ventilation, no change was observed in gill Na^+/K^+-ATPase activity during the 48-h anoxia exposure, suggesting that branchial ion permeability was unaffected. We conclude that rapid "channel arrest" of NMDA receptors likely prevents excitotoxicity in the brain of the goldfish, and that a more slowly developing decrease in Na^+/K^+-ATPase activity also contributes to the profound metabolic depression seen in these animals during oxygen starvation.

1. Introduction

The freshwater turtles *Chrysemys picta* and *Trachemys scripta*, crucian carp (*Carassius carassius*), and goldfish (*Carassius auratus*) are four of the most anoxia-tolerant vertebrates known, and are capable of surviving days or weeks without oxygen at low temperatures (see Lutz and Nilsson, 1997; Nilsson, 2001; Bickler and Buck, 2007 for reviews). However, the strategies used by these animals to survive anoxia are distinct. The turtles become dormant as they overwinter for up to 4 months at the bottom of lakes, ponds or streams, without oxygen (Jackson et al., 1984). In contrast, the crucian carp and goldfish remain active during prolonged anoxia, albeit at reduced activity levels, which provides them with the opportunity to seek out oxygenated waters (Nilsson et al., 1993; Rausch et al., 2000).

During anoxia, these facultative anaerobes reduce their ATP demands to a degree greater than that predicted by temperature-dependent metabolic depression. Further, anoxic ATP production proceeds solely by glycolysis which leads to the generation of lactate and metabolic acid (Jackson et al., 1984; Lutz and Nilsson, 1997; Bickler and Buck, 2007). The turtle is capable of tolerating very high lactate and metabolic acid loads, much of which is buffered by the shell (Jackson et al., 1984). The crucian carp and goldfish, on the other hand, avoid such "self-pollution" (Lutz and Nilsson, 1997) by transporting the lactate to the muscle where it is converted into ethanol via alcohol dehydrogenase (Shoubridge and Hochachka, 1980; Nilsson, 1988) and excreted across the gills (Shoubridge and Hochachka, 1980; Johnston and Bernard, 1983; Nilsson, 1991).

In most vertebrate neurons, anoxia starves ion motive transporters, such as the Na^+/K^+-ATPase, of ATP. As a result transmembrane ion gradients breakdown, causing neurons to depolarize due to the loss of intracellular K^+, and gain of intracellular Na^+ and Ca^{2+}. The breakdown of ion gradients results in the excess release of neurotransmitters such as glutamate, which contributes to overactivation of NMDA receptors, further increasing intracellular Ca^{2+} concentration, and the activation of proteases which contribute to cytoskeletal breakdown. Further,

☆ Contribution to the Special Issue of CBP on Chinese Comparative Biochemistry and Physiology presented at or related to the International Conference of Comparative Physiology, Biochemistry and Toxicology and the 6th Chinese Comparative Physiology Conference, October, 10–14, 2007, Zhejiang University, Hangzhou, China.

* Corresponding author. Tel.: +1 519 884 0710x3313; fax: +1 519 746 0677.
E-mail address: mwilkie@wlu.ca (M.P. Wilkie).

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doi:10.1016/j.cbpc.2008.06.004

increases in [Ca^{2+}] promote the generation of reactive oxygen species (ROS) during subsequent re-oxygenation/re-perfusion and further neuron damage. Together, NMDA receptor overactivation, loss of ion gradients, and ATP depletion result in cell swelling, membrane blebbing and finally necrosis. These events are collectively termed "excitotoxicity" (see Nilsson, 2001; Mishra et al., 2001; Sattler and Tymianski, 2000; Walsh et al., 2007 for reviews).

Under anoxic conditions, ATP may be conserved by reducing the permeability of the plasma membrane to ions through "channel arrest" (Hochachka, 1986), which would decrease the ATP requirements of ion motive pumps such as the Na^+/K^+-ATPase. In neurons, channel arrest of excitatory receptors permeable to Ca^{2+} would also minimize the risk of excitotoxic cell death caused by excess intracellular Ca^{2+} accumulation (Nilsson, 2001; Bickler and Buck, 2007). In the red-eared slider (*T. scripta*) anoxia-induced decreases in the abundance of voltage gated Na^+ channels (Pérez-Pinzón et al., 1992), reduced K^+ permeability in brain (Chih et al., 1989), and reduced Na^+/K^+-ATPase activity in brain (Hylland et al., 1997) and in hepatocytes (Buck and Hochachka, 1993) are indicative of channel arrest. The only direct measures of channel arrest are the reduction in NMDA receptor, and more recently AMPA (alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor, currents reported in anoxic turtle cortical sheets using single-channel and/or whole-cell patch-clamping (Buck and Bickler, 1998; Bickler et al., 2000; Shin and Buck, 2003; Shin et al., 2005; Pamenter et al., 2008).

There is little support for the channel arrest hypothesis in crucian carp or goldfish (Lutz and Nilsson, 1997). However, there is evidence of metabolic depression under anoxic conditions using a crucian carp brain slice model (Johansson et al., 1995), and respective reductions in the sensitivity of visual (Johansson et al., 1997) and auditory nerves (Suzue et al., 1987; Fay and Ream, 1992) have been reported during anoxia and hypoxia in crucian carp and goldfish. The goal of the present study was to examine channel arrest of the NMDA receptor during *in vitro* anoxia in goldfish telencephalon slices using the whole-cell patch-clamp technique. Because ethanol is known to interfere with mammalian NMDA receptor function (Lovinger et al., 1989), another objective was to test the hypothesis that the ethanol inhibits NMDA receptors during anoxia. In a second series of experiments, we also measured Na^+/K^+-ATPase activity in the brains and gills of goldfish exposed to anoxia *in vivo*, to determine if other mechanisms of ion channel arrest might be used during longer-term (48 h) oxygen starvation.

2. Methods and materials

2.1. Experimental animals and holding

Large goldfish (common comets; *C. auratus*; 50–100 g) used in patch-clamp studies were purchased from a commercial supplier (Aleong International, Mississauga, Ontario) in the summer–autumn of 2003 and 2004 and held in flowing dechlorinated City of Toronto tap water at 15–20 °C at the University of Toronto. Smaller goldfish (5–10 g) were purchased in late autumn 2006 and used for *in vivo* experiments investigating the effects of anoxia on Na^+/K^+-ATPase activity in the brain and gills. The smaller fish were held in flowing (flow rate ~ 1–2 L min^{-1}), aerated (dissolved oxygen > 90% saturation), well water [composition (in mmol L^{-1}) Na^+ ~ 0.8; Cl^- ~ 0.5; Ca^{2+} ~ 3; pH 8.0; temperature 12 ± 1 °C] in 110-L tanks at Wilfrid Laurier University. All fish were fed three times weekly with appropriately sized commercial pellets, but the smaller fish were starved for 1 week prior to *in vivo* anoxia exposure experiments to minimize the effects that build-ups of nitrogenous waste could have on their response to oxygen starvation.

2.2. Experimental protocols

2.2.1. Whole-cell patch-clamping of goldfish brain slices

The whole brain of the large goldfish was removed from the cranium following decapitation of the fish and immediately placed in a solution of oxygenated and chilled (4 °C) artificial cerebrospinal fluid [aCSF; Composition (in mmol L^{-1}): NaCl 107, KCl 2.6, NaH_2PO_4 2.0, $NaHCO_3$ 26.5, glucose 20, HEPES–Na 10.0, $MgCl_2$ 1.0, $CaCl_2$ 1.2, osmolality 280–290 mOsm; pH 7.6]. Both lobes of the telencephalon were then dissected away from the main body of the brain while in chilled aCSF solution, and temporarily stored in a vial (15 mL) of oxygenated aCSF on ice. Within 15 min, each telencephalon was affixed to a sectioning block using cyanoacrylate glue ("Krazy Glue"), and submerged in ice-cold aCSF contained in the reservoir of a Vibtratome 1000 tissue slicer (Vibratome, St. Louis, MO, USA). Tissue slices were then cut in the parasaggital plane (300 μm thick; 3–4 for each lobe). Slices were gently lifted out of the reservoir using a fine paint brush and transferred to a vial of oxygenated aCSF and stored for up to 48 h. Preliminary experiments revealed that the slices remained electrophysiological viable, and capable of generating action potentials and NMDA receptor currents, for up to 48 h following their preparation.

Individual telencephalon slices were placed on a coverslip contained in a flow-through perfusion chamber (RC-26, Warner Instruments, Hamden, CT, USA) and held in place by a horseshoe-shaped stainless steel slice hold-down anchor across which strands of lycra thread were stretched at 2-mm intervals (Warner Instruments). The chamber was gravity perfused with oxygenated or anoxic aCSF (at room temperature — 22–23 °C) from a 1.0-L glass bottle via an intravenous (IV) dripper. A fast-step drug perfusion system (VC-6 Perfusion System, Warner Instruments) was used to deliver tetrotodotoxin (TTX; 1 μmol L^{-1}), *N*-methyl-D-aspartate (NMDA; 300 μmol L^{-1}), or other drugs to the slice (e.g. MK801, ethanol; see below) during whole-cell patch-clamp recording experiments.

Whole-cell patch recordings were made using 2–5-MΩ pipettes, into which a Ag–AgCl electrode was connected to a CV-4 headstage and AxoPatch-1D amplifier. The electrolyte solution in the recording electrode was composed of (in mmol L^{-1}) NaCl 8, $CaCl_2$ 0.0001, HEPES–Na 10, KCl 20, potassium gluconate 110; $MgCl_2$ 1, NaGTP 0.3; NaATP 2 (pH 7.4). Gigaohm seals (5–10 GΩ) were established by advancing the recording electrode towards the slice in μm increments using a "stepper-motor". A whole-cell configuration was established by voltage-clamping the recording electrode potential to −60 mV, and applying a sharp pulse of suction. The resting membrane potential was then recorded by switching from the voltage clamp to zero-current setting of the amplifier. Resting membrane potentials typically ranged from −50 to −70 mV. Data were collected using a TL-1 DMA interface (Axon Instruments) connected to the amplifier, and digitized and stored on a personal computer with Clampex 6 software (Axon Instruments).

2.2.2. Measurement of NMDA receptor currents and effects of MK801 and Mg^{2+}

NMDA receptor currents were measured from slices pre-perfused with TTX for 10 min prior to NMDA application. TTX suppressed action potentials that could interfere with NMDA receptor current measurement. Neurons were voltage clamped at −70 mV and NMDA applied to the preparation until a current response was noted (usually 5–10 s). The current response was followed for 5 min, when the amplifier was switched back to zero-current mode from voltage-clamp mode.

The irreversible NMDA receptor antagonist — MK801, and high doses of Mg^{2+} (4 mmol L^{-1}) were also applied to block/inhibit NMDA receptor currents and confirm that we were measuring NMDA receptor-mediated currents. Because MK801 is an open channel NMDA receptor antagonist, the slice was pre-perfused with the blocker for 10 min and a second NMDA receptor current was initiated to allow the drug to penetrate and block the open channel. Additional NMDA receptor recordings were then made at 10 min intervals to confirm an effective blockade. High concentrations of Mg^{2+} reversibly abolish NMDA receptor currents. Accordingly, NMDA receptor currents were measured in slices bathed in aCSF containing 0.1 mmol L^{-1} Mg^{2+}, followed by 10 min of perfusion with 4 mmol L^{-1} Mg^{2+} to confirm blockade of the channel.

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2.2.3. Effects of anoxia on NMDA receptor currents

The telencephalon slices were exposed to either aCSF gassed with oxygen (95% O_2/5% CO_2) or anoxic aCSF gassed with nitrogen (95% N_2/5% CO_2). Control experiments comprised measurements of NMDA receptor currents at 20-min intervals during 60–80 min exposures of the slices to the oxygenated aCSF. The effects of anoxia were studied by measuring control currents in oxygenated aCSF, followed by measurement of NMDA receptor currents at 20-min intervals during a 40-min exposure to the anoxic aCSF. Anoxic conditions in the set-up were confirmed in separate trials by withdrawing sub-samples of perfusate from the recording chamber and measuring the dissolved oxygen with an OM2000 oxygen electrode and meter (Cameron Instruments, Port Aransas, TX, USA).

2.2.4. Effects of ethanol on NMDA receptor currents

As goldfish are known to produce ethanol in millimolar quantities under anoxic conditions (Shoubridge and Hochachka, 1980) we also measured NMDA receptor currents in slices exposed to 10 mmol L^{-1} ethanol for 40 min. As in the anoxia experiments, recordings were first made in ethanol-free oxygenated aCSF, followed by a 40-min exposure to ethanol-loaded aCSF.

2.2.5. Effects of anoxia on brain and gill Na^+/K^+-ATPase activity

In these experiments, small goldfish were subjected to anoxia for a period of 48 h, following a two-week acclimation at the experimental temperature of 8 °C. The night before experiments, small goldfish in groups of 6–8 were transferred to individual 2-L Erlenmeyer flasks covered by a mesh screen. The entire apparatus was then submerged in a water bath at the experimental temperature of 8 °C, and gently aerated. Anoxia was established by filling each flask to overflow with water, and bubbling the water with compressed N_2 until the dissolved O_2 concentration in the flask was less than 1% of saturation (10–15 min). The chamber was then sealed with a rubber stopper and submerged in the water bath to ensure that air could not enter the chamber water from the outside. Care was taken to ensure that there were no gas bubbles trapped in the flask following the insertion of the stopper.

Gill ventilation rates were recorded at regular intervals (0.25, 0.5, 1, 2, 3, 4, 8, 12, 24, 36, 48 h) under anoxic conditions, and following 24 h recovery in normoxic water. Sub-sets of fish were then anesthetized with tricaine methane sulfonate (MS222; 0.5 g L^{-1} buffered with 1.0 g L^{-1} $NaHCO_3$) under control (normoxic) conditions, after 4, 8, 12, 24 or 48 h of anoxia, and following a 24-h recovery from 48 h of anoxia. The brain was immediately removed from these fish, as well as the second and third gill arches, and snap frozen in liquid N_2. Both gill and brain were stored at −80 °C until analyzed for Na^+/K^+-ATPase activity.

2.2.6. Measurement of Na^+/K^+-ATPase activity

Analysis of both brain and gill Na^+/K^+-ATPase activity was based on the methodology of McCormick (1993). Brain samples were thawed on ice, and transferred to an ice-cold 1.5-mL centrifuge tube to which 500 μL of ice-cold homogenization buffer comprised of 3 parts SEI buffer (composition in mmol L^{-1}: sucrose = 150, EDTA = 10, imidazole = 50), and 1 part SEID buffer (composition: 1.0 g sodium deoxycholate per L SEI buffer) was added. The brain tissue was then homogenized for 30 s on ice using a hand-held Teflon pellet pestle. The resulting mixture was then centrifuged at 5000 ×g to remove insoluble material and connective tissue, the supernatant was then diluted a further five times using ice-cold homogenization buffer. Individual gill filaments were processed in a similar manner, but unlike brain no further dilution of the homogenate was needed.

In all cases Na^+/K^+-ATPase measurements were made within 15 min of homogenization in 96-well microtiter plates which were analyzed on a Spectramax190 plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Unless indicated otherwise, all reagents were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). The Na^+/K^+-ATPase activity of the brain was calculated by subtracting ouabain-inhibited ATPase activity from total ATPase activity of tissue homogenates measured simultaneously on the plate spectrophotometer. The background ATPase activity therefore represented the remaining ATPase activity in the tissue homogenate after ouabain inhibition. Gill Na^+/K^+-ATPase was determined in a similar manner, but rather than inhibiting the Na^+/K^+-ATPase with ouabain, K^+ was omitted from the reaction buffer to deprive the Na^+/K^+-ATPase of its extracellular substrate (K^+) because the gills of goldfish are relatively insensitive to ouabain (Chasiotis and Kelly, 2008).

For each experiment, two reagent mixtures were used. The first was an enzyme mixture comprised of imidazole buffer (50 mmol L^{-1}; pH 7.5) containing phospho*enol*pyruvate (2.8 mmol L^{-1}), ATP (3.5 mmol L^{-1}), NADH (0.2 mmol L^{-1}), lactate dehydrogenase (4 units mL^{-1}), and pyruvate kinase (5 units mL^{-1}). The second was a salt solution comprising imidazole (50 mmol L^{-1}, pH 7.5), NaCl (189 mmol L^{-1}), $MgCl_2$ (10.5 mmol L^{-1}) and KCl (42 mmol L^{-1}). As described above, KCl was omitted, and replaced with an equivalent amount of NaCl, from the salt solution when background ATPase activity was determined on gill samples only. A reaction buffer was prepared from 3 parts enzyme solution and 1 part salt solution immediately prior to ATPase activity measurements, and kept on ice.

For determination of total ATPase activity and background ATPase activity, 25 μL of freshly prepared gill or brain homogenate was added to each well of the microtiter plate (in triplicate for each respective measurement), which was kept chilled on an ice pack wrapped in paper towel. This was followed by the addition of 200 μL of the freshly prepared reaction mixture to each well. The plate was then transferred to the plate spectrophotometer, and the rate of NADH oxidation (disappearance) was measured at 340 nm and 25 °C.

Because ATPase activity was expressed in μmol ADP per min, it was necessary to quantify ADP in the tissue homogenates analyzed. Accordingly, an ADP standard curve was constructed using assay medium to which known concentrations of ADP were added, which resulted in the linear decreases in absorbance (at 340 nm) due to the oxidation of NADH in the assay medium. The protein concentration of both gill and brain homogenates was determined spectrophotometrically at a wave length of 750 nm using a commercial kit (Lowry Assay, Sigma-Aldrich), so that Na^+/K^+-ATPase activity could be expressed as μmol ADP mg^{-1} protein min^{-1}.

2.2.7. Calculations and statistics

In whole-cell patch-clamp recording experiments, the initial NMDA receptor current under oxygenated conditions was set to 100%, and subsequent currents (either control or anoxia exposure) were normalized to this value (Shin and Buck, 2003).

Total ATPase activity in brain and gills was calculated by dividing the rate of decrease in the absorbance of the reaction media by the slope of the ADP standard curve to yield ATPase activities in μmol ADP min^{-1}. These ATPase activities were then divided by the protein concentration of each sample (mg protein) to yield total and background ATPase activities in μmol ADP mg^{-1} protein min^{-1}. The Na^+/K^+-ATPase activity was the difference between total ATPase activity minus background ATPase activity according to the following equation:

$$Na^+/K^+\text{-ATPase activity} = \text{Total ATPase activity} - \textit{Background ATPase activity}. \quad (1)$$

Background ATPase activity was that remaining after the Na^+/K^+-ATPase activity was eliminated using either ouabain (for brain) or omitting K^+ from the reaction buffer (for gills; see above).

All respiration rates, Na^+/K^+-ATPase activity measurements, and actual and normalized NMDA receptor currents were expressed as the mean ± 1 standard error of the mean (SEM). Significant differences were determined using one-way analysis of variance (ANOVA), and

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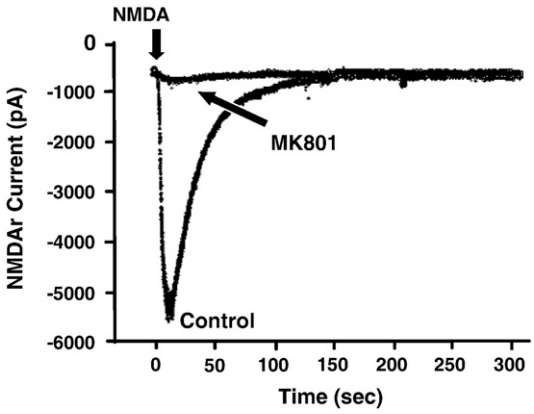

Fig. 1. Effects of MK801 on NMDA receptor (NMDAr) currents in the goldfish telencephalon. Representative raw current traces showing the effect of the open channel blocker MK801 on whole-cell NMDA receptor currents in the goldfish (*Carassius auratus*) telencephalon. Application of MK801 (50 μM) completely abolished NMDAR currents normally elicited by application of 300 μM NMDA to the slice preparation ($N=5$ separate experiments).

where significant variation was observed, statistical differences between the means were determined using the Tukey–Kramer post-test at the $P<0.05$ level. Where the mean data compared had unequal variances, statistical analysis was performed using a Kruskal–Wallis test followed by Dunn's Multiple Comparison's post-test at the $P<0.05$ level. All statistical analysis was performed with GraphPad InStat, Version 3.02 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. In vitro NMDA receptor function under normoxic and acute anoxic conditions

The average membrane potential of normoxic neurons measured in the goldfish telencephalon was −58.8±2.6 mV ($N=25$; data not shown). Application of NMDA resulted in the generation of NMDA receptor currents with an average amplitude of 2185±310.5 pA ($N=25$; data not shown), which were completely and irreversibly blocked by application of the open channel NMDA receptor blocker — MK801 (Fig. 1). Moreover, NMDA receptor currents were inhibited by more than 80% following exposure to high concentrations of Mg^{2+} (4 mmol L^{-1}) relative to control slices containing low Mg^{2+} concentrations (0.1 mmol L^{-1}; Fig. 2A, B). Anoxia caused an immediate reduction in normalized NMDA receptor current amplitude after 20 min (Fig. 3A), and the attenuation persisted over 40 min (Fig. 3A). When data were pooled from several anoxia trials, the mean normalized NMDA receptor currents were 40–50% lower in anoxic compared to normoxic control slices measured in oxygenated saline (Fig. 3B). There was no change in the membrane potential of neurons during anoxia (−62.0±3.6 mV; $N=10$; data not shown). Exposure of the telencephalon slices to aCSF containing 10 mmol L^{-1} ethanol for 40 min under oxygenated conditions, resulted in no significant change in NMDA receptor current amplitude (Fig. 4A, B).

3.2. In vivo responses to anoxia over 48 h

All goldfish withstood 48 h *in vivo* anoxia exposure at 8 °C, but two of eight fish that were re-introduced to normoxic water died before

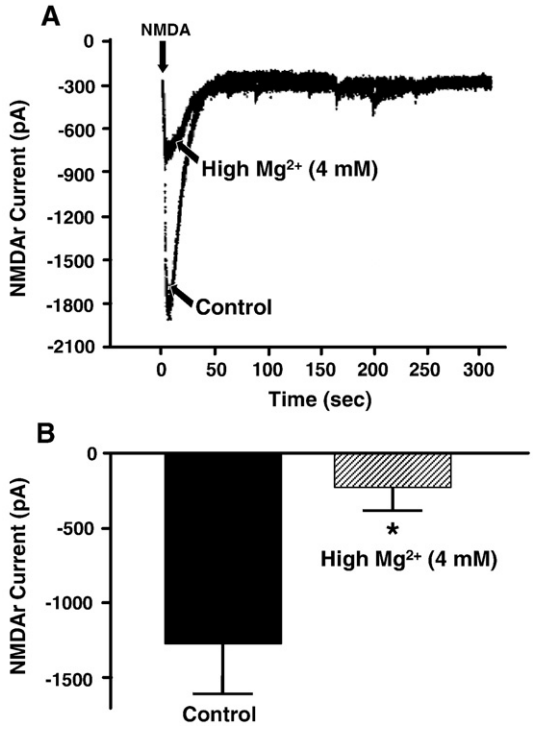

Fig. 2. Effects of Mg^{2+} on NMDA receptor currents in the goldfish telencephalon. (A) Representative raw current traces illustrating NMDA receptor current amplitude in the same slice preparation during exposure to control aCSF (0.1 mmol L^{-1} Mg^{2+}) or aCSF containing high Mg^{2+} (4 mmol L^{-1} Mg^{2+}). (B) Mean NMDA receptor current amplitudes measured in goldfish telencephalon slices during exposure to control aCSF or aCSF containing high Mg^{2+} ($N=5$ separate experiments). All data are presented as the mean±1 SEM. Asterisk denotes significant differences between the control and high Mg^{2+} treatments.

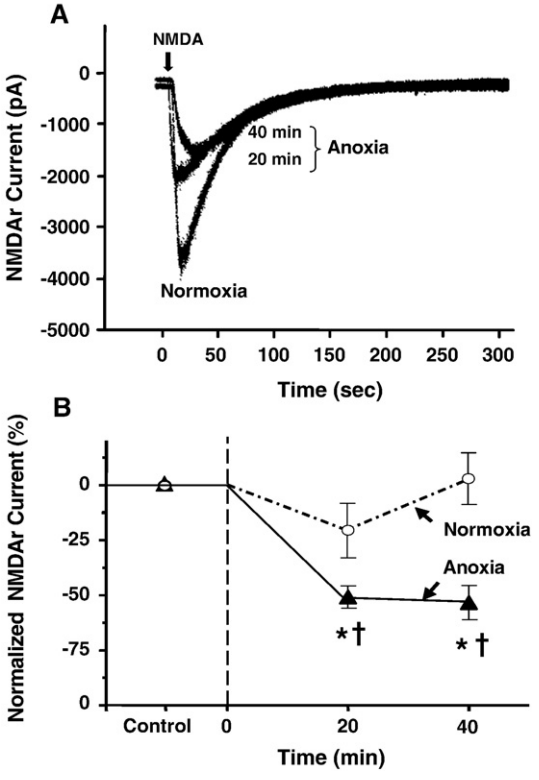

Fig. 3. Effects of 40 min anoxia on NMDA receptor currents in goldfish telencephalon. (A) Raw current traces illustrating the change in NMDA receptor current amplitude following 20 and 40 min anoxia. (B) Mean normalized NMDA receptor currents under normoxic conditions ($N=5$ at each measurement period; open circles) or during a 40-min perfusion with anoxic aCSF ($N=5$ at each measurement period; triangles). All data are presented as the mean±1 SEM. Asterisks denote significant differences from pre-anoxia exposure conditions, while daggers denote significant differences from corresponding normoxic controls.

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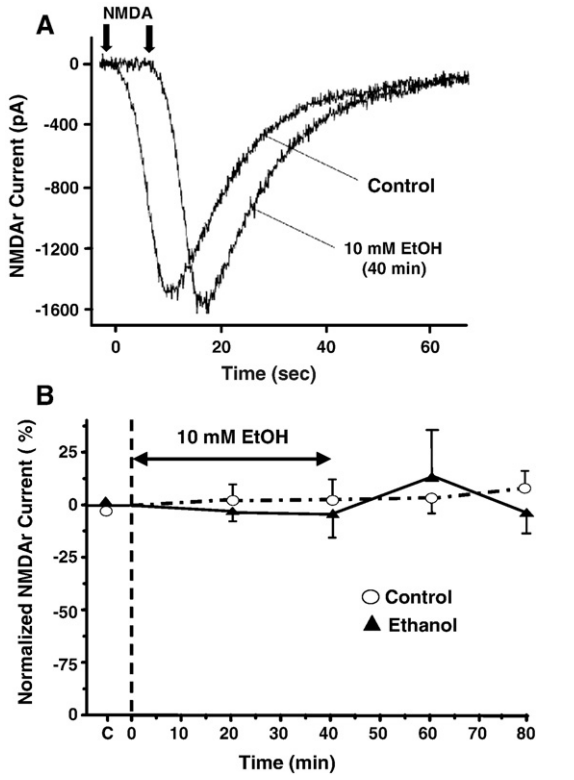

Fig. 4. Effects of ethanol (10 mmol L^{-1}) on NMDA receptor currents in goldfish telencephalon. (A) Representative raw current traces from the same slice before (control) and after perfusion with ethanol enriched aCSF (40 min). (B) Mean changes in normalized NMDA receptor currents under normoxic conditions (N=6–7 at each measurement period; open circles) or during a 40-min perfusion with ethanol (N=4–5 at each measurement period; triangles) enriched aCSF (arrow) followed by perfusion with ethanol-free aCSF. All data are presented as the mean±1 SEM.

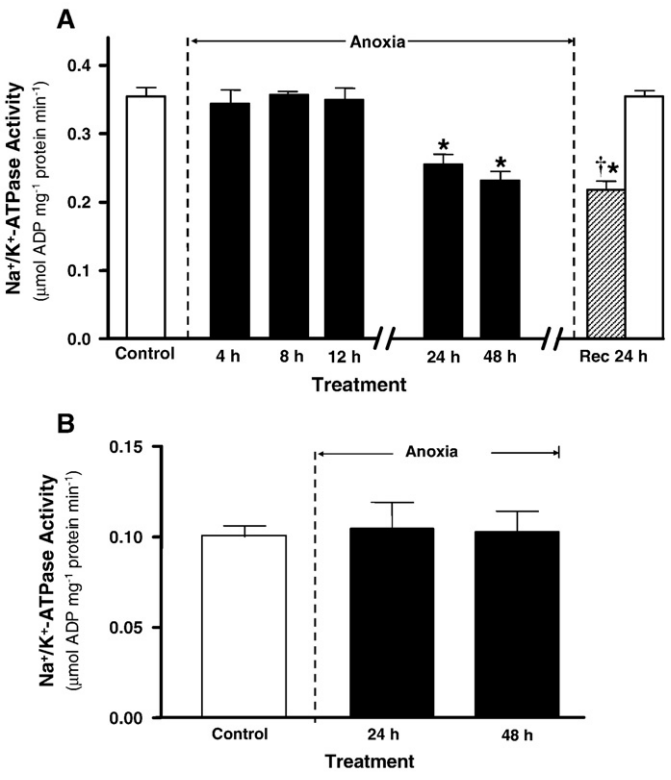

Fig. 6. Effects of anoxia on brain and gill Na^+/K^+-ATPase activity in goldfish. (A) Na^+/K^+-ATPase activity in whole brain, under normoxic (control) conditions (open bars; N=8–10) or over 48 h of anoxia (solid bars; N=6), followed by a 24-h recovery in normoxic water (hatched bars; N=7). (B) Na^+/K^+-ATPase activity measured in gill under normoxic (control) conditions (open bars; N=10) or after 24 or 48 h of anoxia (solid bars; N=6). All data are presented as the mean±1 SEM. Asterisks denote significant differences from normoxic control fish, and dagger denotes significant difference between fish recovering from anoxia in normoxic water from simultaneous normoxic controls.

the end of the 24-h recovery period. Survival under anoxia was characterized by a marked reduction in activity level (swimming), and by 48 h a loss of equilibrium (balance). The decrease in activity during *in vivo* anoxic exposure was accompanied by decreased rates of ventilation. There was an immediate 23% reduction in ventilation rate after 15 min, and an overall decline of 40% by 30 min. By 4 h of anoxic exposure, ventilation rate was reduced by 50% compared to control fish held under normoxic conditions. Ventilation rate continued to steadily decline with anoxia and by 24 h it was reduced by 70%, and more than 90% by 48 h (Fig. 5). Ventilation rate only recovered to 50% of the normoxic rate following 24 h recovery from anoxia (Fig. 5).

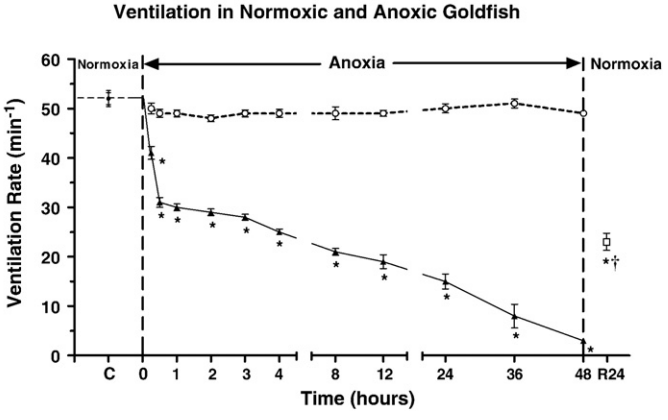

Fig. 5. Effects of anoxia on gill ventilation in goldfish. Ventilation rate (opercular beats) measured under normoxic (open circles, broken line) and anoxic (triangle, solid line) conditions, and after 24 h recovery from anoxia (open square). Because fish were terminally sampled as the experiments proceeded, sample size decreased with time. Data are presented as the mean±1 SEM. Under anoxic conditions N=16 from 0–12 h, N=4–6 at 24 and 32 h, and N=3 at 48 h, and under normoxic conditions N=6–10 from 0–12 h, and N=4 at 24–48 h. For fish recovering from anoxia N=6. Asterisks denote significant differences from normoxic animals. Dagger denotes a significant difference from anoxic fish at 48 h.

Brain Na^+/K^+-ATPase activity was unaltered during the first 12 h of anoxia. After 24 and 48 h of anoxia, however, the respective rates of Na^+/K^+-ATPase activity were 27% and 35% lower than rates measured in fish held under normoxia (Fig. 6A). The Na^+/K^+-ATPase activity remained depressed, by approximately 35%, following 24 h recovery in normoxic water (Fig. 6A). There was no anoxia-induced suppression of Na^+/K^+-ATPase activity in the gills (Fig. 6B).

4. Discussion

4.1. Channel arrest of the NMDA receptor

Goldfish NMDA receptor currents are blocked by high [Mg^{2+}] and MK801 just as mammal and turtle NMDA receptors are. These observations provide strong evidence that the currents measured here can be ascribed to NMDA receptors. NMDA receptors have been described previously in the goldfish nervous system (Smeraski et al., 1999) and in other teleosts (e.g. Harvey-Girard and Dunn, 2003; Tzeng et al., 2007). The goldfish telencephalon, from which we made our recordings, also contains NMDA receptors (Xu et al., 2003), making it an ideal preparation upon which to study the response of NMDA receptor currents to anoxia.

The decrease in NMDA receptor activity during *in vitro* anoxic exposure is the first direct evidence that channel arrest occurs in the goldfish as a defense against oxygen starvation. Goldfish NMDA

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receptor currents were attenuated during anoxia in the same manner first reported in the western painted turtle using a similar whole-cell patch-clamp protocol (Shin and Buck, 2003), and using single-channel patch-clamp (Buck and Bickler, 1998). The NMDA receptor is a ligand-gated cation channel that is highly permeable to Ca^{2+} and activated by glutamate (see Mishra et al., 2001; Wenthold et al., 2003 for reviews). Potentiation of this receptor is involved in learning and memory in vertebrates, but its overactivation due to excess glutamate release during anoxia or hypoxia causes pronounced increases in intracellular Ca^{2+}, which contributes to excitotoxic cell death (Mishra et al., 2001; Nilsson, 2001; Bickler and Buck, 2007; Walsh et al., 2007). An ability to resist excitotoxicity through the rapid/immediate attenuation of NMDA receptor currents could therefore be important for surviving the initial phase of anoxia in goldfish (Buck and Bickler, 1998; Bickler et al., 2000; Shin and Buck, 2003; Shin et al., 2005).

Channel arrest of the NMDA receptor may at least partially explain the reduced auditory and visual nervous system sensitivity that has been reported in anoxic goldfish and crucian carp, respectively (Suzue et al., 1987; Fay and Ream, 1992; Johansson et al., 1997). It is notable that NMDA receptors play key roles in modulating these systems (e.g. Vandenbranden et al., 2000; Wolszon et al., 1997), so an ability to attenuate NMDA receptor responsiveness during anoxia might be consistent with decreased sensory activity in the anoxic goldfish. Consistent with decreased neuronal activity is a general 40% decrease in metabolic activity in anoxic crucian carp brain slices as measured by microcalorimetry (Johansson et al., 1995). As pointed out by Nilsson (2001) an ability to maintain a minimum level of nervous system activity provides crucian carp and goldfish with the opportunity to seek out oxygenated waters, but energy savings can be realized by reducing the activity of different regions of the nervous system. Unlike the western painted turtle and red-eared sliders, which become virtually comatose during anoxia, anoxic crucian carp remain active, but at reduced levels (Nilsson et al., 1993). Thus, NMDA receptor channel arrest may contribute to some of the nervous system adjustments reported in anoxic crucian carp and goldfish. The mechanism(s) that initiate attenuation of the NMDA receptor current during anoxia are unclear, however.

It is possible that the decrease in NMDA receptor currents we observed during anoxia were triggered by changes in the state of receptor phosphorylation. In the western painted turtle and in mammals, phosphorylation of the NMDA receptor NR1 sub-unit increases Ca^{2+} currents, and dephosphorylation decreases currents (Wenthold et al., 2003; Bickler and Buck, 2007). Inhibition of the protein phosphatases PP1 and PP2A in anoxic turtle cortical neurons blocked the attenuation of NMDA receptor currents during anoxia (40 min), providing evidence that reductions in NMDA receptor currents were related to dephosphorylation of the receptor (Bickler et al., 2000; Shin et al., 2005). It remains to be determined if a similar mechanism explains the reduction in goldfish NMDA receptor currents in anoxic goldfish brain. Based on our findings we can suggest that the attenuation of NMDA receptor currents observed in the goldfish and turtle during anoxia represents a common strategy to prevent excitotoxic cell death during O_2 starvation.

Ethanol might be an additional means of attenuating NMDA receptor currents in the anoxic goldfish. During anoxia, both goldfish and crucian carp tissues generate ATP via anaerobic glycolysis resulting in the accumulation of pyruvate, and subsequently lactate. However, the lactate is taken-up by the white and red muscles where it is converted to ethanol via alcohol dehydrogenase (ADH) (Johnston and Bernard, 1983; Nilsson, 1988). Tissue and blood ethanol concentrations in both fish may approach 5–7 mmol L^{-1} during brief bouts of anoxia (Johnston and Bernard, 1983; Rausch et al., 2000), which is sufficient to inhibit NMDA receptors in mammalian neurons (Lovinger et al., 1989; Hoffman et al., 1989; Popp et al., 1999). However, NMDA receptor currents were unaltered in the presence of ethanol, suggesting that the goldfish NMDA receptor is resistant to this metabolic byproduct of anoxia.

Tolerance to ethanol might be important when goldfish are hypoxic or anoxic, when nervous system activity is reduced (Nilsson and Renshaw, 2004). Because ethanol is a general nervous system depressant, the possibility that it is interfering with other neurophysiological processes in goldfish cannot be discounted. However, it is notable that Rausch et al. (2000) noted that brain ethanol (~4 mmol L^{-1}) was not related to the development of hypoactivity in the goldfish, further supporting our contention that the nervous system of these animals may be resistant to physiologically relevant concentrations of ethanol.

4.2. Effects of longer-term anoxia on Na^+/K^+-ATPase activity in brain and gills

In the western painted turtle Na^+/K^+-ATPase activity is reduced by more than 30% during 24 h of anoxia (Hylland et al., 1997) and we demonstrate a similar strategy may be used by the goldfish under anoxic conditions. The Na^+/K^+-ATPase maintains the electrochemical gradient across neuronal cell membranes and it is estimated to consume approximately 60% of the total ATP turnover (Bickler and Buck, 2007). Thus, considerable energy savings may result through decreases in ion channel permeability or "ion channel arrest" and subsequent decreases in Na^+/K^+-ATPase activity (Hochachka, 1986).

In contrast to the goldfish, no change in Na^+/K^+-ATPase activity was reported in wild crucian carp captured in ponds during winter (February) and subjected to anoxia for a period of 6 weeks at 4 °C (Vornanen and Paajanen, 2006). Due to seasonal and temperature acclimatization, Na^+/K^+-ATPase activities in the crucian carp were already substantially depressed to 10% of rates measured during the summer. As Vornanen and Paajanen (2006) point out, ion channel arrest under such conditions was not likely needed when Na^+/K^+-ATPase activity was already profoundly depressed. Because the goldfish in this study were domesticated and held in the lab at warmer temperatures (8–12 °C), we suggest that they were less likely to have undergone such pre-acclimation/acclimatization to anoxia. It would be useful to determine if crucian carp exposed to anoxia at warmer summer temperatures undergo similar decreases in Na^+/K^+-ATPase activity as we report in goldfish. While Hylland et al. (1997) did expose crucian carp to anoxia at warmer temperatures (20 °C) these fish did not undergo a decline in Na^+/K^+-ATPase activity. One possibility for this difference from our study is that the period of anoxia was insufficient (24 h) to cause a change in Na^+/K^+-ATPase activity. Because crucian carp are more anoxia tolerant than goldfish (Nilsson and Renshaw, 2004; Bickler and Buck, 2007), it may be less urgent for this fish to initiate the drastic ATP conservation strategy of reducing ion pumping, which would be accompanied by a lowering of the brain's electrical activity. We therefore propose that the reduction we observed represents a second, more slowly developing response to anoxia when the need to conserve ATP becomes critical, such as could occur if glycogen or ATP stores were depleted to low levels. Indeed, the high glycogen content of crucian carp and goldfish livers is thought to be essential for anoxic survival (Nilsson, 1990).

Unlike the painted turtles, which readily recover from short anoxic episodes with a complete restoration of Na^+/K^+-ATPase activity (Hylland et al., 1997), Na^+/K^+-ATPase activity remained suppressed during post-anoxia recovery in the goldfish. Hypoxia, ischemia and post-hypoxia (ischemia) recovery in mammals is associated with pathological reductions in Na^+/K^+-ATPase activity, which is thought to result from damage to the Na^+ and K^+ binding sites by reactive oxygen species (ROS), and greater phosphorylation of the enzyme (Graham et al., 1993). In anoxic goldfish, such damage seems unlikely because the generation of ROS would not occur in the total absence of oxygen. However, there may have been oxidative damage to Na^+/K^+-ATPases during the subsequent 24-h period of re-oxygenation in goldfish. Vornanen and Paajanen (2006) observed a 30% decrease in Na^+/K^+-ATPase activity in crucian carp during recovery from prolonged anoxia which they attributed to post-anoxic ROS generation. Moreover, Lushchak et al. (2001) reported an increase in conjugated dienes during post-anoxia re-oxygenation in goldfish.

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It was notable that there was no change in gill Na^+/K^+-ATPase activity during anoxia. At first glance, the gill seems like a logical target for channel arrest because of the need to maintain ATP turnover to drive branchial ion exchange (Marshall, 2002). However, total gill Na^+/K^+-ATPase activity only represents a small proportion (~1%) of total oxygen consumption in teleosts (Morgan and Iwama, 1999) making it less likely that appreciable energy savings could result from channel arrest. Further, net ion movements across the gill may have been unaffected by anoxic exposure.

At relatively cool temperatures, there is very little protrusion of the lamellae from the gill filaments of normoxic crucian carp (15 °C) and goldfish (7.5 °C), and therefore a low lamellar exposure area (Sollid et al., 2005a). During hypoxia, however, the gills of crucian carp are remodelled, increasing the functional lamellar surface area several fold, presumably to increase the gas exchange surface area, which is also accompanied by greater ion losses (Sollid et al., 2003) as a result of osmorespiratory compromise (Gonzalez and McDonald, 1992; Nilsson, 2007). However, such remodelling is not observed under anoxic conditions (Sollid et al., 2005b), which would be futile when there is no oxygen available. Because the fish used in our experiments were acclimated to relatively cool (8 °C) oxygenated water, we speculate that they likely lacked protruding lamellae (cf. Sollid et al., 2005a). As a result, the goldfish would have had no need to remodel the gill during anoxia, which would have caused no corresponding change in ion losses, and hence no change in branchial Na^+/K^+-ATPase activity.

5. Summary and conclusions

Although the brain of the goldfish and crucian carp does not completely "shut-down" during anoxia or hypoxia, the weight of evidence indicates that there is a substantive decrease in metabolic rate (Johansson et al., 1995), and a decreased activity in sensory regions of the nervous system (Johansson et al., 1997; Fay and Ream, 1992). The present study demonstrates that in the early acute phase of anoxia, there is a rapid arrest of NMDA receptor activity, which may protect against excitotoxicity and contribute to overall ATP conservation. A more slowly developing decrease in Na^+/K^+-ATPase activity may also be triggered in the brain but not the gill, which could contribute further to the partial metabolic depression of nervous system activity that characterizes the anoxic response of the goldfish.

Acknowledgements

We thank Michael Richards and Valeria Ramaglia for their assistance with these experiments, and along with the Wilfrid Laurier University (WLU) Animal Care Staff, assisting in the care of the goldfish. This work was funded by NSERC Discovery Grants to MPW and LTB. LTB was also the recipient of a Premier's Research Excellence Award. The research of SA and DC was supported by the WLU Science and Technology Endowment Program (STEP). This work was originally presented at the International Conference of Comparative Physiology, Biochemistry and Toxicology & 6th Annual Chinese Comparative Physiology Conference, Hangzhou, China. We gratefully acknowledge the financial support of WLU and the Social Sciences and Humanities Research Council (SSHRC) in the form of a travel grant, which enabled MPW to attend this meeting.

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