GABA receptor inhibition and severe hypoxia induce a paroxysmal depolarization shift in goldfish neurons

Nariman Hossein-Javaheri and Leslie Thomas Buck
Department of Cell & Systems Biology, University of Toronto, Toronto, Ontario, Canada

Abstract

Mammalian neurons undergo rapid excitotoxic cell death when deprived of oxygen; however, the common goldfish (Carassius auratus) has the unique ability of surviving in oxygen-free waters, under anoxia. This organism utilizes γ-amino butyric acid (GABA) signaling to suppress excitatory glutamatergic activity during anoxic periods. Although GABAA receptor antagonists are not deleterious to the cellular survival, coinhibition of GABAA and GABAB receptors is detrimental by abolishing anoxia-induced neuroprotective mechanisms. Here we show that blocking the anoxic GABAergic neurotransmission induces seizure-like activity (SLA) analogous to a paroxysmal depolarization shift (PDS), with hyperpolarization of action potential (AP) threshold and elevation of threshold currents. The observed PDS was attributed to an increase in excitatory postsynaptic currents (EPSCs) that are normally attenuated with decreasing oxygen levels. Furthermore, for the first time, we show that in addition to PDS, some neurons undergo depolarization block and do not generate AP despite a suprathreshold membrane potential. In conclusion, our results indicate that with severe hypoxia and absence of GABA receptor activity, telencephalic neurons of C. auratus manifest a paroxysmal depolarization shift, a key feature of epileptic discharge.

NEW & NOTEWORTHY This work shows that the combination of anoxia and inhibition of GABA receptors induces seizure-like activities in goldfish telencephalic pyramidal and stellate neurons. Importantly, to prevent seizure-like activity, an intact GABA-mediated inhibitory pathway is required.

action potential; membrane potential; patch-clamp electrophysiology; seizure; whole cell conductance

INTRODUCTION

With termination of oxidative phosphorylation due to decreasing oxygen levels, the brain resorts to glycolysis as the only major source of ATP production. This strategy cannot meet the metabolic demands of neurons, and ATP shortage leads to excitotoxic cell death (1). Although true for the majority of vertebrate species, not all are vulnerable to low oxygen levels. The crucian carp (Carassius carassius) and the common goldfish (Carassius auratus) are among the most anoxia-tolerant vertebrates known and can survive under severe hypoxia for weeks to months (1, 2). Of particular interest is their ability to tolerate oxygen lack without apparent neuronal damage (1). The brain consumes 20% of the body's oxygen to produce ATP, most of which is utilized for maintaining the ion gradients and membrane potential following neuronal firing (3, 4). Yet, anoxia-tolerant organisms are capable of reducing the metabolic demands of the brain by lowering ATP synthesis and demand by up to 90% (1). Attenuation of ATP turnover is achieved through a decrease in the neuronal activity via a number of overlapping mechanisms, namely, “channel arrest,” “spike arrest,” and “synaptic arrest” (5–7). In brief, these strategies aim to reduce energetically demanding neuronal excitation when ATP is scarce and oxidative phosphorylation is not possible.

GABAergic signaling is one of the major pathways that contribute to neuronal survival during anoxia by suppressing cellular excitability. In the absence of O2, extracellular GABA concentrations are doubled in the telencephalon of crucian carp (8, 9). Meanwhile, neuronal membrane potential shifts toward a depolarizing GABAergic reversal potential through a “shunting inhibition” mechanism in goldfish neurons. Although depolarizing, this mechanism simultaneously suppresses excitatory pyramidal neurons and stimulates the...
inhibitory stellate cells (10). Disruption of GABA signaling is deleterious to cellular survival and leads to irreversible damage (11). Therefore, without the protective effects of GABA, these neurons are incapable of tolerating anoxia and undergo excitotoxicity: excessive glutamate exposure and disruption of glutamate/GABA ratio that causes cellular swelling, irreversible neuronal injury, and eventually cell death (12).

While recording the electrical activity of goldfish telencephalic neurons following inhibition of GABA<sub>A</sub> receptors, we noticed unusual action potentials that deviated from the expected patterns: depolarization followed by a hyperpolarization ± afterhyperpolarization (10). Particularly of interest, were apparent seizure-like activities (SLA) and a paroxysmal depolarization shift (PDS) in these neurons. PDS are unregulated variability in the membrane potential with a clear depolarization plateau following the discharge of multiple action potentials (APs) (13). The plateau may last anywhere from ten to several hundred milliseconds before hyperpolarization occurs and termination of the refractory period (13, 14). A combined reduction in GABAergic and increased glutamatergic neurotransmission is largely responsible for the generation of PDS. In this situation, the excitatory postsynaptic potentials (EPSPs) can no longer be regulated and lead to PDS formation (15). PDS has been observed in goldfish Mauthner cells with GABA<sub>A</sub> receptor antagonism using pentylenetetrazol (16) but never under anoxic conditions. Because the telencephalon of teleost fish and rodent hippocampi have certain structural and functional similarities (17), experimentation on this region of the cortex allows for more relevant cross-species comparisons. In this study we report that goldfish neurons show PDS and abnormal fluctuations in cellular membrane voltage that stem from disinhibition (inhibition of GABAergic neurotransmission) during anoxia.

**METHODS**

**Animals**

Goldfish (*Carassius auratus*), weighting 50–100 g, were purchased from AQUAlity Tropical Fish Wholesale (Mississauga, ON, Canada). They were kept in freshwater flow-through tanks filled with dechlorinated, sand-filtered, City of Toronto tap water at 18°C under 12-h light-dark cycles. This study was approved by the University of Toronto Animal Care Committee and was designed according to the relevant guidelines issued by the Canadian Council of Animal Care used for experimentation.

**Dissection and Tissue Preparation**

Following decapitation, and removal of the top of the cranium, the brain of the unanesthetized goldfish was removed from the cranium using a scoopula and was placed in chilled (4°C) artificial cerebrospinal fluid (aCSF) with the following composition: 20 mM NaHCO<sub>3</sub>, 118 mM NaCl, 1.2 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.9 mM KCl, 10 mM HEPES-Na, 10 mM d-Glucose and 2.4 mM CaCl<sub>2</sub> (pH 7.6, 280–290 mOsm). Within an hour of dissection, the telencephalon was cut and separated from the rest of the brain, and each hemisphere was sliced into 300-μm-thick slices using a Leica VT1200S (Leica Microsystems Canada, Concord, ON) vibratome while still in ice-cold aCSF. Slices were stored at 4°C in oxygenated aCSF and were used within a period of 48 h. Slices remained physiologically viable within this period and were reliable for electrophysiological studies (18).

**Patch-Clamp Electrophysiology**

Individual slices were placed in a RC-26 perfusion chamber (Harvard Apparatus Canada, Saint-Laurent, QC), filled with aCSF (composition described in Dissection and Tissue Preparation Section) and were initially perfused with room-temperature (22°C) aCSF bubbled with 99% O<sub>2</sub> and 1% CO<sub>2</sub>. The 1% CO<sub>2</sub> was chosen to mimic fish blood pCO<sub>2</sub> and the balance of the tank was O<sub>2</sub> 99%. High O<sub>2</sub> levels were used to overcome the loss of vascular O<sub>2</sub> supply, and some O<sub>2</sub> is lost to the atmosphere above the recording chamber. We therefore do not know what the O<sub>2</sub> concentration is inside the slice, although it is likely hyperoxic, and we define this as the normoxic control condition. Two bottles of aCSF were placed ~30 cm above the recording chamber. A three-way stopcock and flow controller (FR-50 Harvard Apparatus Canada, Saint-Laurent, QC, Canada) were used to select between the normoxic aCSF and anoxic aCSF and to control the flow rate at ~2–3 mL/min. To elicit an anoxic response, the stopcock was switched so that aCSF would flow from the bottle bubbled with 99% N<sub>2</sub>/1%CO<sub>2</sub>. Whole-cell recordings were performed using the voltage clamp method with 9–11 MΩ borosilicate glass pipette electrodes (Harvard Apparatus Canada) containing (in mM) 120 K-gluconate, 10 KCl, 15 sucrose, 2 Na<sub>2</sub>ATP, 0.44 CaCl<sub>2</sub>, 1 EGTA and 10 HEPES-Na (pH 7.6 adjusted with KOH; osmolarity, 285–290 mOsm). The electrode was connected to a CV-4 headstage (Gain: 1/100 U, Axon Instruments, Sunnyvale, CA) and an AXOPATCH 1D patch-clamp amplifier (Axon Instruments). A whole-cell patch configuration was established by voltage-clamping the recording potential to ~60 mV and by applying negative pressure after a 1–20 GΩ seal was established. All seals were obtained using the blind-patch technique (19). Following whole-cell capacitance compensation, access resistance (R<sub>a</sub>) or whole-cell resistance was 30–40 MΩ. The recording and the treated slice were discarded if R<sub>a</sub> changed by 10–15 MΩ. Action potential threshold (AP<sub>th</sub>) was determined by injecting current in steps (1 nA) until an AP was elicited. Recordings were obtained from both pyramidal and stellate telencephalic neurons, and each cell was identified electrophysiologically as described previously (20, 10). In short, pyramidal neurons demonstrate rapid adaptation in response to a suprathreshold current, whereas stellate cells showed no accommodation in response to the same stimulus.

**Pharmacology**

Telencephalic slices were perfused with pharmacological agents through a fast-step perfusion system (VC-6 perfusion valve controller and SF-77B fast-step perfusion system; Harvard Apparatus Canada). Before perfusion, pharmacological compounds were dissolved in aCSF and bubbled with 99% N<sub>2</sub> and 1% CO<sub>2</sub> to ensure perfusion did not reoxygenate the severely hypoxic slice. To block the GABAergic responses, GABA<sub>A</sub> and GABA<sub>B</sub> receptor antagonists, 25 μM SR95531 gabazine (GZ), 100 μM bicuculline (BIC), and 5 μM CGP-54648 (CGP) were used under severely hypoxic conditions. Voltage-
gated sodium channels were blocked with 1 μM tetrodotoxin (TTX). All chemicals were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) except CGP-55848 and (TTX). All chemicals were obtained from Sigma-Aldrich acid (APV) purchased from Sigma-Aldrich Canada Ltd.

**Statistics**

Statistical analyses were performed using SigmaPlot (version 11.0; Systat Software, Inc., San Jose, CA). All data were tested for normality and equal variance and analysis of variance using repeated-measures ANOVA for between-group comparisons where appropriate. Post hoc analyses used *t* tests for within-subject comparisons and Games–Howell’s test for between-subject designs. Significance was determined at *P* < 0.05. Results are presented as means ± SE unless otherwise specified, and *n* equals the number of animals used to calculate a mean value.

**RESULTS**

**Perfusion Chamber Reaches Severely Hypoxic Levels Sufficient for Anoxic Brain Injury**

The critical oxygen tension (PCrit) is the oxygen partial pressure at which oxidative metabolism can no longer support the energetic (ATP) needs of the cell (21). To ensure that the aCSF flowing through the perfusion chamber contained [O2] equivalent to anoxia when bubbled with 99% N2 and 1% CO2, an oxygen sensor, Loligo System Witox 1 Oxygen Analyzer with D212 Dipping probe (Viborg, Denmark), was used to measure O2 levels (PO2) in the chamber. With normoxia (99% O2 and 1% CO2) to anoxia transition, oxygen levels in the chamber rapidly decreased, and within 20 min, PO2 reached ~6 mmHg and stabilized (Fig. 1). PO2 values <40 mmHg are considered severely hypoxic and <20 mmHg is the threshold for anoxic brain injury in the mammalian brain (22). Anoxia-tolerant fishes, such as carp, are capable of tolerating lower PCrit in a temperature-dependent manner; however, at standard room temperature, critical oxygen tension for carp is also 20 mmHg (23). Further anoxic treatment did not reduce PO2 any further than 6 mmHg and complete anoxia (PO2 = 0 mmHg) even after 30 min was never achieved. Because PO2 < 20 mmHg can cause anoxic brain injury, and we were able to reach PO2 as low as 6 mmHg, therefore, the term “severely hypoxic” is used, and we equate this with PO2 sufficient to elicit an “anoxic response.” Furthermore, although oxygenation with 99% O2 is not physiological and the tissue is hyperoxic, this approach was a means to supply the sample with O2 and overcome the loss of vascular O2 delivery. Because our baseline was at 99% O2 saturation, this state was referred to as “normoxic” for simplicity.

**PDS Observed with GABAA and GABAB Receptor Inhibition**

Although not identified in the Carassius auratus brain, both synaptic and extrasynaptic GABAA receptors are present in the crucian carp (Carassius carassius) brain (24). Owing to close relationship between the two species, it is reasonable to assume that goldfish also express both GABAA receptor subtypes. In a previous study, we reported that when exposed to severe hypoxia without any pharmacological manipulations, membrane potential of both stellate and pyramidal neurons shift toward a depolarizing GABAA reversal potential (EgABA), inducing an inhibitory shunt in pyramidal cells only; this depolarization sufficiently generates action potentials in the inhibitory stellate cells, suppressing the overall electrical activity of the brain (10). In this experiment, we observed that the application of both 100 μM bicuculline (Fig. 2, A and B) and 25 μM gabazine (Fig. 2, C and D) reversed the anoxia-induced membrane depolarization and the “shunting inhibition” but did not generate SLA in either cell types (n = 3 for stellate neurons and n = 3 for pyramidal neurons). Because gabazine is more potent than bicuculline at blocking GABA currents (25), we decided to use gabazine in our experiment.

With both GABAA and GABAB receptor inhibition, goldfish telencephalic neurons displayed SLA in two distinct patterns. Approximately 50% of our recordings (n = 5 recordings) underwent uncontrolled depolarization and continuous action potential firing beyond the point of recovery until the patch was lost even though access resistance was not compromised. Therefore, it was suspected that the loss of whole-cell patch configuration was equivalent to neuronal death (Fig. 2, E and F). Morphologically, both stellate and pyramidal cells clearly showed epileptogenic patterns of paroxysmal depolarization shift (PDS) with giant depolarizing potentials (GDPs); action potentials of decreasing amplitude, a depolarization plateau, and termination by repolarization followed by an afterhyperpolarization until the next PDS (Fig. 2Ea and 2Eb) for stellate and pyramidal neurons respectively; please note that these traces are enlarged sections of Fig. 2, E and F (14). A combined analysis of pyramidal and stellate neurons showed that severely hypoxic action potential (AP) threshold hyperpolarized from −29.69 ± 4.33 mV to −44.85 ± 2.49 mV with GABA receptor inhibition (n = 5–7, *P* < 0.001; Fig. 2G). In addition, AP threshold current was also reduced from 4.84 ± 0.8 nA to 1.6 ± 0.4 nA (n = 5, *P* < 0.001; Fig. 2H). Under normal

![Figure 1](https://example.com/figure1.png) Changes in oxygen tension during a normoxic-anoxic-normoxic transition. The lowest obtained PO2 was ~6 mmHg. The gray arrow indicates the time at which the chamber’s gas composition was switched to 99% N2 and 1% O2. The black arrow indicates the initiation of reoxygenation (99% O2, 1% CO2).
physiological conditions, stellate and pyramidal neurons have distinct electrophysiological responses to anoxia (10); however, inhibition of GABAergic signaling significantly compromises the survival ability in all neurons regardless of their type, making their unique approach to anoxia tolerance irrelevant. Under such conditions, pyramidal and stellate neurons cannot be distinguished electrophysiologically. Therefore, recorded data from these cells were analyzed as a single group.
Not All Neurons Undergo Excitotoxic Cell Death with GABA Receptor Inhibition

Although blocking the GABA_A receptors alone is not detrimental to the cell, inhibition of both GABA_A and GABA_B receptors interrupts cellular survivability with anoxia. Yet, ~50% of goldfish telencephalic neurons in our recordings did not undergo cell death with inhibition of GABA signaling (n = 5 for both stellate and pyramidal neurons) (Fig. 3, A and B). These cells maintain a depolarized membrane potential following GABAergic inhibition with an increased action potential potentiality and SLA. Seizure-like activities manifested the same patterns of a PDS: an escalated increase in the depolarizing membrane potential with continued burst of repetitive action potentials that are maintained for several hundred milliseconds followed by hyperpolarization. Yet, despite constant epileptiform responses and suprathreshold potential with each AP firing, GABA receptor inhibition alone was not sufficient to induce cell death. Within minutes of gabazine and CGP application and SLA, cells became quiescent (Fig. 3, A and B). At this stage, electrical activity was suppressed and membrane potential returned to baseline. Duration of SLA was variable between cells aside from the fact that no cell tolerated continuous activity longer than 5 min; however, all cells demonstrated a shutdown where pretreatment current injection failed to initiate an action potential and APs were generated only if suprathreshold stimulation with a higher current was administered (Fig. 3, C and D). It was therefore speculated that owing to intense cellular excitation, these pyramidal and stellate cells undergo accommodation and demonstrate depolarization block (26). Both cell types were subjected to injecting current (I) in 1 nA steps until an AP was elicited. During severe hypoxia and before GABA receptor inhibition, I = 5.6 ± 1.67 nA (n = 5) was sufficient to generate an action potential. With depolarization block, the necessary current almost tripled to I = 16.4 ± 8.41 nA (n = 5, P < 0.05; Fig. 3E). To ensure that our recordings were not a result of artifacts, a voltage-gated sodium channel blocker, 1 μM tetrodotoxin (TTX) was used to prevent AP generation (n = 3) (Fig. 3F).

Excitatory Neurotransmission is Enhanced with GABA Receptor Inhibition during Severe Hypoxia

PDS is the hallmark of cellular epilepsy and is induced by GABA receptor blockage, or disinhibition (27). Following disinhibition, excitatory neurotransmission dominates, which is perhaps the major cause of anoxia-induced SLA observed in the goldfish telencephalon neurons. Similar to Wilkie et al. (18), we show that with severe hypoxia, EPSCs mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptors are suppressed in pyramidal neurons. To closely observe current activity, cells were voltage-clamped at −75 mV and exposed to severe hypoxia. The amplitude of excitatory (NMDA/AMPA) currents during normoxia was −249.45 ± 13.1 pA and decreased to −116.75 ± 32.1 pA with severe hypoxia. To ensure that these were indeed excitatory currents, NMDA and AMPA receptor antagonists (CNQX 25 μM and APV 25 μM) were applied. With pharmacological manipulation, these currents were significantly suppressed and reached an amplitude of −6 ± 3.36 pA, whereas the cells were still severely hypoxic (n = 3, P < 0.001); yet, application of 100 μM bicuculline increased current amplitude to −181.74 ± 15.1 pA (n = 3; Fig. 4, A–D). No significant difference was observed between normoxia and severely hypoxic + bicuculline (P = 0.07). In this experiment we were able to confirm that under normal physiological conditions, EPSCs are suppressed with severe hypoxia. However, if tonic and phasic GABA_A receptor signaling is pharmacologically blocked during severe hypoxia exposure, EPSCs are no longer inhibited and the arrest of NMDA and AMPA receptors that protects against neural excitotoxicity is disrupted. Therefore, GABAergic signaling is essential for the augmentation of glutamatergic neurotransmission under anoxic conditions without which cells lose the ability to regulate excitability that can be manifested as SLA.

DISCUSSION

With oxygen deprivation, ATP synthesis in mammalian neurons halts and the activity of Na^+/K^+ ATPase pumps is inhibited. Unable to maintain ion gradients, membrane potential depolarizes and excitatory neurotransmission (dominantly via glutamate and aspartate) is initiated. If continued, Ca^{2+} influx through NMDA channels activates intracellular Ca^{2+}-dependent signaling cascades that eventually leads to excitotoxic cell death (28, 29). Meanwhile, goldfish (Carassius auratus) and crucian carp (Carassius carassius) can survive prolonged periods of severe hypoxia through both behavioral and physiological modifications (1). At a neuronal level, cellular survival is dependent on GABAergic signaling as it has been shown in another anoxia-tolerant organism, the western painted turtle (Chrysemys picta bellii) (11). In this article, we report that through blockade of both GABA_A and GABA_B receptors, goldfish telencephalic neurons demonstrate seizure-like activities consistent with a paroxysmal depolarization shift. In mammalian neurons, PDS can emerge from GABA_A receptor inhibition alone using picrotoxin or bicuculline (30, 31). However, bicuculline-induced PDS is recorded only if fast excitatory neurotransmission through AMPA and NMDA receptors is simultaneously present (32). This supports our experiments and explains why bicuculline was not sufficient to induce PDS. Owing to “channel arrest,” AMPA and NMDA receptor currents decreased by at least 50% in goldfish neurons (18). Although not fully

Figure 2. Protective anoxic responses are GABA-dependent. In the absence of GABAergic signaling, cells undergo seizure-like activity (SLA). 100 μM bicuculline (BIC) and 25 μM gabazine (GZ) reverses both inhibitory stellate (A and C) and excitatory pyramidal (B and D) cellular responses during anoxia to preanoxic stages; however, suppression of GABA_A receptors alone does not cause SLA in either cell type (n = 3 for each cell type treated with BIC or GZ). Excitotoxicity and SLA in pyramidal (E) and stellate (F) neurons with paroxysmal depolarization shift during anoxia and GABA_A/B receptor inhibition. Recordings marked a and b are the expanded sections of E and F, respectively. G: changes in the action potential (AP) threshold (mV) pre- and post-GABA_A/B receptor inhibition. H: changes in AP threshold current (nA) pre- and post-GABA_A/B receptor inhibition. Data presented in G and H are combined analysis from pyramidal and stellate neurons (n = 5–7 per treatment, ***P < 0.001). Treatments were 25 μM gabazine (GZ) and 5 μM CGP-55848 (CGP) under anoxic conditions [99% N_2, 1% CO_2-bubbled artificial cerebrospinal fluid (aCSF)]. Data are means ± SE.
Figure 3. Depolarization block in stellate (A) and pyramidal (B) neurons. Antagonizing both GABA$_{A/B}$ receptors produces seizure-like activity (SLA) until complete suppression of neural activity and AP firing ($n = 5$). a/b: enlarged current-clamped traces demonstrating depolarization blocks from A and B. Treatments including 25 μM SR95531 gabazine (GZ), and 5 μM CGP-55848 (CGP) were used under anoxic conditions [99% N$_2$, 1% CO$_2$-bubbled artificial cerebrospinal fluid (aCSF)]. Pyramidal (Ci/ii) and stellate (Di/ii) neuronal responses before and after GABA receptor inhibition during anoxia. E: both cell types showed an increase in the current necessary for action potential (AP) firing following the suppression of GABAergic signaling and depolarization block ($n = 5$, *$P < 0.05$). F: observed APs were 1μM tetrodotoxin (TTX)-sensitive, suggesting that all recordings represented true action potentials. Data are means ± SE.
understood, it is possible that current attenuation under anoxia is due to changes in the phosphorylation status of AMPA and NMDA receptors. In the western painted turtle, blocking protein phosphatases PP1 and PP2A following 40 min of anoxia inhibits NMDA receptors, indicating that channel arrest is regulated by phosphorylation/de-phosphorylation of these receptors (33, 34). Assuming goldfish utilize a similar mechanism, then either the duration and/or the strength of GABAA receptor suppression is not adequate to completely undo the attenuation of excitatory currents and promote the formation of PDS. Hence, a greater degree of excitation is required to compromise the integrity of anoxic neurons.

Our 20-min response time to severe hypoxia in goldfish brain slices is likely reflective of the natural decrease in goldfish brain metabolic rate, but, to the best of our knowledge, this has not been directly measured. Using direct calorimetry, a 70% decrease in metabolic rate in response to anoxia w a sm e a s u re di ni s o l a dg o l d- fi sh brain sheets, but the exposure to anoxia was several hours before measurements were made (35, 36). In anoxia-tolerant turtle brain slices, a metabolic depression was measured within a 20- to 30-min time frame using direct calorimetry (37); and in whole goldfish, metabolic heat production decreased within 20 min of reaching the critical oxygen tension for oxidative metabolism (38).

Our data also suggest that inhibition of GABAB receptors is required for PDS. These G-protein-linked receptors modulate K⁺ or Ca²⁺ channels via a second messenger phospholipase C (PLC) and adenylyl cyclase (AC)/cAMP-dependent pathway (39). Only a few studies have focused on the direct role of GABAB receptors in PDS. Blocking of GABAB receptors directly reduces the amplitude of PDS afterhyperpolarization in rats through a combination of GABAB response and Ca²⁺-dependent K⁺ currents (40, 41). In mammals, presynaptic activation of GABAB receptors inhibits the influx of Ca²⁺ via voltage-dependent Ca²⁺ channels at the synaptic boutons.

Figure 4. Current recordings of goldfish telencephalic neuron (voltage clamped at −75 mV) during normoxia and anoxia. A: with anoxia, the amplitude to excitatory currents decreased and were completely abolished with the application of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists [(2R)-amino-5-phosphonovaleric acid/6-cyano-7-nitroquinoxaline 2,3-dione (APV/CNQX)]. B: application of GABA receptor inhibitor, bicuculline (BIC), increased current amplitude to preanoxic levels and prevented the anoxia-induced current suppression. C: enlarged examples of the decrease in excitatory current amplitude with anoxia and APV and CNQX. D: summary data of changes in current amplitudes. Treatments: normoxia [99% O₂, 1% CO₂-bubbled artificial cerebrospinal fluid (aCSF)], anoxia (99% N₂, 1% CO₂-bubbled aCSF). [CNQX] = 25 μM, [APV] = 25 μM and [BIC] = 100 μM. Data are means ± SE. *P < 0.05, **P < 0.001. n = 3 per treatment. n.s., nonsignificance.
Ca\(^{2+}\) is required for vesicle fusion and release and, as a result, activation of these receptors inhibits synaptic neurotransmission (42). Therefore, GABA\(_B\) receptors contribute to neuronal inhibition by preventing glutamate release (43). This is evident in the anoxic turtle brain where application of CGP during anoxia increases action potential frequency and increases AMPA receptor-mediated EPSPs (11). Although not studied yet, it is possible that GABA\(_A\) receptors become the primary regulator of neuronal energy consumption during severe hypoxia and GABA\(_A\) inhibition. Hypoxic-ischemic encephalopathy in mice, is detected by AMP-activated protein kinase (AMPK), a sensor of cellular stress in response to ATP shortage (44). AMPK suppresses ATP consumption and stimulates ATP-generating pathways instead, leading to an overall metabolic depression (45). In anoxic crucian carp neurons, phosphorylation of AMPK is greatly elevated, and although not shown in goldfish, AMPK directly regulated the activity of GABA\(_A\) receptors and activate inwardly rectifying K\(^-\) channels (46, 47). Yet, inhibition of GABA\(_A\) receptors alone is not deleterious to severely hypoxic goldfish neurons (10). Therefore, it appears that GABA\(_B\) receptors become significantly more important if GABA\(_A\) receptors are not functional.

In addition to PDS, we were able to show for the first time that goldfish telencephalic neurons show depolarization block (DB) and this held true for \(-50\%\) of our recordings. DB results from changes in extracellular ion concentrations, particularly hyperkalemia with high-frequency stimulations. With an elevated extracellular [K\(^+\)], cells are unable to repolarize, K\(^+\) channels remain open, and depolarization is prevented (48). Although action potentials are absent, DB is by no means “inhibitory,” as continuous AP firing is not necessary for prolonging seizures at a cellular level (25). In this period, some Ca\(^{2+}\) channels remain open and excitatory neurotransmission can continue (49). Overall, 30\%–50\% of the brain ATP is consumed to maintain membrane potential through Na\(^+\)/K\(^+\) ATPases (50). To conserve energy, the activity of these pumps is reduced by 30\%–40\% in goldfish neurons with anoxia (18). Therefore, because the termination of GABAergic signaling with severe hypoxia compromises the protective machinery of the cell, regulated activity of Na\(^+\)/K\(^+\) ATPases should also fail. As a result, extracellular [K\(^+\)] increases, which mimics the conditions necessary for generation of a depolarization block. Although this phenomenon has been reported previously in rats (51), this is the first time it has been investigated in an anoxia-tolerant organism.

Although in all of our recordings severely hypoxic neurons demonstrated uncontrolled excitability, it is not clear why some displayed PDS, while others showed DB. This observation might be attributed to the neuronal network present in a telencephalic slice. Generally, two types of PDS discharges are categorized in neurons: endogenous and network-driven. Endogenous bursts are independent of other cells, while a group of neurons is required for the generation of network-driven bursts (14). Meanwhile, as reported in rats, DB is primarily network-driven and is regulated based on the interaction between pyramidal and inhibitory interneurons in hippocampal slices (52). In our study, the telencephalon was cut into three to four slices per hemisphere during preparation for patch-clamping. Therefore, it is possible that the integrity of the neural network was compromised, and cells underwent DB depending on the presence or absence of intact circuitry.

Other cellular pathways might also contribute to PDS in the severely hypoxic neurons. For instance, formation of reactive oxygen species (ROS) incites epileptic seizures in the mammalian brain (53, 54). With severe hypoxia, ROS production and intracellular [ROS] are suppressed. Scavenging ROS during anoxia increases the whole-cell NMDA currents by 100\% and also promotes direct GABA release from inhibitory interneurons in the anoxia-tolerant turtle, which does not happen normally (55, 56). This suggests that ROS exposure might be a key regulator for anoxic protection in addition to GABAergic signaling.

We conclude that the combination of severe hypoxia and inhibition of GABA receptors induces seizure-like activities in goldfish telencephalic pyramidal and stellate neurons. These activities were recorded as either paroxysmal depolarization shifts or depolarization blocks and were attributed to a direct increase in cellular excitation and EPSCs upon suppression of GABAergic signaling. Without functional GABA\(_A\) and GABA\(_B\) receptors, anoxia tolerance is lost and neuronal survival is impaired. Therefore, to prevent seizure-like activities in these cells, an intact GABA-mediated inhibitory pathway is required.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

#### AUTHOR CONTRIBUTIONS

L.T.B. conceived and designed research; N.H-J. performed experiments; N.H-J. analyzed data; N.H-J. interpreted results of experiments; N.H-J. prepared figures; N.H-J. drafted manuscript; L.T.B. edited and revised manuscript.

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