Long-lasting effects of chemical hypoxia on spinal cord function in tadpoles

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We investigated the effects of chemical hypoxia on the central pattern generator controlling swimming in stage 42 *Xenopus laevis* larvae. We recorded motoneuron activity from ventral roots of immobilized tadpoles and evoked swim episodes by brief electrical stimulation of the tail skin. In the presence of the metabolic inhibitor, sodium azide (5 mM, NaN_3), swim episode duration and cycle frequency decreased until swim motor patterns could not be evoked. On recovery, cycle frequency returned to preazide levels; however, episode duration remained short for at least an hour. In addition, recovery induced spontaneous, short bouts of swimming similar to the slow rhythm that is evoked by *N*-methyl-D-aspartic acid. We conclude that abiotic features of the environment can have long-term modulatory effects on circuit function in the CNS. *NeuroReport* 21:943–947 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Environmental stressors, such as hyperthermia and hypoxia have acute effects on neural function [1], potentially leading to arrest or inappropriate activity. Mechanisms exist to shift optimal ranges for molecular operation [2] and thus, preserve cellular function, but relatively little is known about phenotypic plasticity at the level of neural circuits, which could optimize behavior. There is increasing evidence that the sensitivity of central circuits to ambient conditions can be tuned by cellular signaling pathways. For example, the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP)/protein kinase G pathway modulates the thermosensitivity of vital central pattern generators (CPG) in animals as diverse as insects (locust [3], drosophila [4]), tadpoles [5], and mice [6]. An explanation for the existence of such modulation is that it optimally configures circuit operation for predictable conditions to conserve energy or prevent overexcitation. This suggests that preexposure to abiotic stressors can have long-lasting adaptive effects on the operation of central circuitry. We tested this idea by examining the effects of chemical hypoxia on the spinal CPG controlling swimming of tadpoles.

Network function and modulation of the swimming CPG in *Xenopus laevis* tadpoles are well described [7,8], and recent studies have investigated the effects of hyperthermia on this circuit [5,9] making it an ideal preparation for our study. Performance of the swimming CPG can be assessed in terms of the duration of swim episodes evoked by electrical stimulation of the tail skin and by the frequency of motor pattern cycles within the episode. These measures are routinely used to monitor the effects of neuromodulators, such as NO and their control mechanisms are relatively well described [10].

Methods

Recording

We used preparations of immobilized, stage 42 [11] *X. laevis* tadpoles obtained from a breeding colony maintained in the School of Biology at the University of St Andrews. Extracellular suction electrodes (approximately 50 μm tip diameter) were used to record motoneuron activity from the ventral roots supplying the trunk muscles (see Ref. [5] for methodological details) and the preparation was bathed in 100 ml of recirculating saline (NaCl 115 mM, KCl 2.5 mM, $NaHCO_3$ 2.5 mM, HEPES 10 mM, $MgCl_2$ 1 mM, $CaCl_2$ 4 mM, pH 7.4 with NaOH). Just suprathreshold electrical stimulation (1 ms pulses at 1.5 × threshold voltage) of the tail skin using another extracellular electrode evoked bouts of centrally generated swimming motor activity (Fig. 1a). Episode duration depends on the length of time without activity, so we stimulated the tail 8 s after the end of the earlier swim episode.

Pharmacology

The chemicals were obtained from Sigma-Aldrich (St Louis, Missouri, USA) and dissolved in distilled water. Sodium azide (NaN_3) inhibits oxidative metabolism by interfering with the electron transport chain at the level of cytochrome *c* oxidase (complex IV) [12], and is commonly used to induce chemical hypoxia in neurons [13–15]. To mimic the effect of a hypoxic environment, we added 5 mM NaN_3 to the standard saline superfusing the

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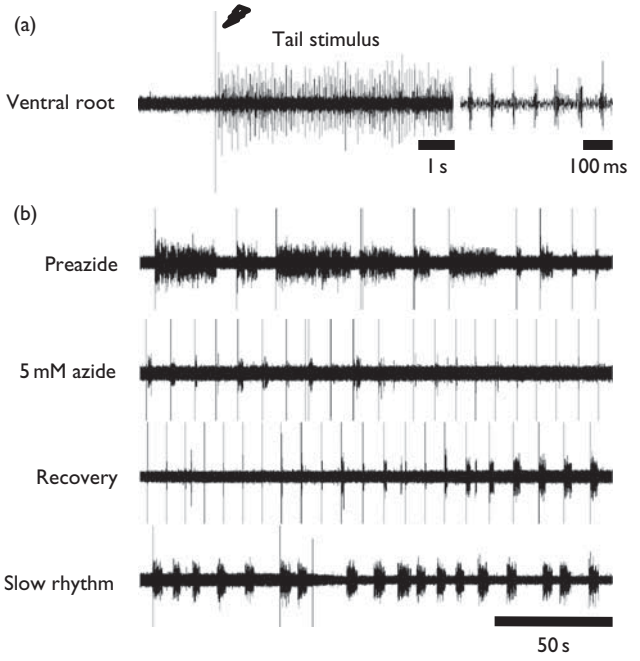
Fig. 1



Chemical hypoxia reduces episode duration and evokes a slow rhythm on recovery. (a) Extracellular recording of motoneuron activity from a ventral root using a glass suction electrode (approximately 50 μm tip diameter) positioned over an intermyotomal cleft. Brief electrical stimulation (1 ms pulses at 1.5 × threshold voltage) using a glass suction electrode applied to the tail skin (tail stimulus, note electrical artifact) activated the swim central pattern generator. Repetitive bursts of motoneuronal activity were monitored in the ventral root recording. The expansion of the trace at the right shows the cycle period to be around 70 ms (cycle frequency = approximately 14 Hz). (b) Preazide – in standard saline tail stimulation evoked swim episodes of variable duration, usually around 30 s. Note that to, standardize the extent of habituation of swimming, 8 s elapsed between the end of a swim episode and the succeeding stimulus. 5 mM azide – in the presence of 5 mM azide swim episodes were reduced in duration and eventually could not be elicited by electrical stimulation. Recovery – superfusion with fresh saline restored the ability to stimulate swimming but swim episode duration remained short. Rhythm – recovery was also associated with the appearance of a spontaneous slow rhythm of repetitive short episodes of swimming. Note swim episodes without electrical stimulation.

preparation. In some experiments, cyanide (1 mM KCN), which also inhibits cytochrome *c* oxidase, was used for comparison. We used 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (100 μM) as a NO scavenger, and *N*-methyl-D-aspartate (NMDA, 50 μM) to evoke a slow rhythm of spontaneous swim episodes.

Data analysis

Motor patterns were digitized using a Digidata 1322A data acquisition system (Molecular Devices, Sunnyvale, California, USA) and displayed and stored using Axoscope 10.1 (Molecular Devices). They were analyzed using Dataview V 6.1 (courtesy W.J. Heitler, University of St Andrews). Graphs were prepared and statistical comparisons were made using Sigmaplot v.11 (Systat Software, Inc., Chicago, Illinois, USA). Data were analyzed for normality and equal variance and appropriate parametric or nonparametric tests were applied as noted in the text. Results were reported as mean ± standard error if the data were distributed normally and as median if otherwise. Significance was assessed with P value of less than 0.05.

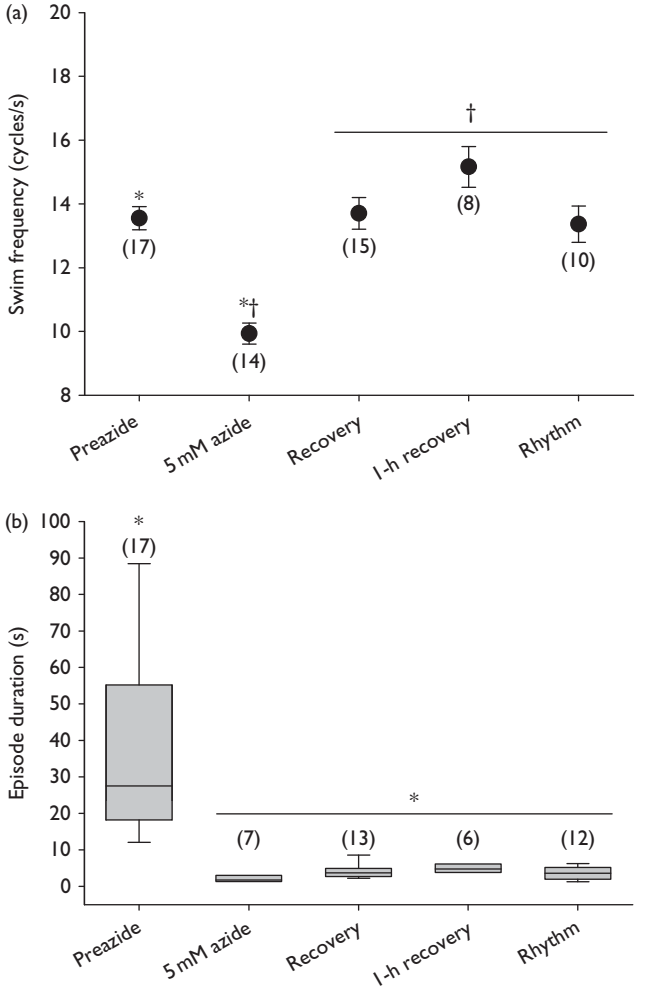
Results

Bath application of 1–2 mM NaN_3 reduced swim episode duration, but was unable to prevent stimulus-evoked motor pattern generation ($n = 3$, not shown). Application of 5–10 mM NaN_3 was effective in stopping swimming, and thus for most experiments we used 5 mM NaN_3 as the minimum reliable dose. Application of 5 mM NaN_3 reduced episode duration dramatically until electrical stimulation was no longer able to elicit swimming (Fig. 1b). Motor pattern arrest occurred in 5.7 ± 0.4 min ($n = 10$). Before azide-induced arrest of central pattern generation, the swim cycle frequency dropped from 13.5 ± 0.4 to 9.9 ± 0.3 Hz (Fig. 2a, statistical treatment provided in figure legend) and the duration of swim episodes decreased from a median of 27.5 to 1.7 s (Fig. 2b).

These effects are similar to the effects of NO on the swim motor pattern [16], and NaN_3 is known to generate NO that may contribute to NaN_3 toxicity independent of its effects on cytochrome *c* oxidase [17,18]. Hence, the effects may be mediated indirectly through NO. However, in the presence of 200 μM of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt, an established NO scavenger that inhibits the effects of NO on the tadpole swim circuit [10], the effect of NaN_3 was unchanged ($n = 4$, Supplemental figure Supplemental digital content 1, *http://links.lww.com/WNR/A83*) indicating that it was not mediated by NO. KCN also inhibits cytochrome *c* oxidase, but is not known to affect the NO/cGMP pathway [17], though it can elevate mitochondrial NO that directly enhances the inhibition of cytochrome *c* oxidase [19]. Perfusion with 1 mM KCN had the same effects on swim episode duration and cycle frequency as NaN_3 ($n = 4$, data not shown) indicating that the effects we describe are most likely the result of inhibition of the electron transport chain in mitochondria and consequent energetic stress.

Washout for 4.3 ± 0.4 min ($n = 10$) in fresh saline was sufficient to restore the ability of electrical stimulation of the skin to evoke swim episodes. The cycle frequency of the restored pattern was 13.7 ± 0.5 Hz and not significantly different from the preazide value and this persisted for at least 1 h at which point the experiments were terminated (cycle frequency with 1 h recovery = 15.2 ± 0.6 Hz) (Fig. 2a). In contrast, the duration of swim episodes did not recover acutely (median episode duration = 3.7 s) or after 1 h (4.8 ± 0.5 s) (Fig. 2b). A striking feature of recovery was the appearance of a slow rhythm of spontaneous episodes of swimming (Fig. 1b). Cycle

Fig. 2

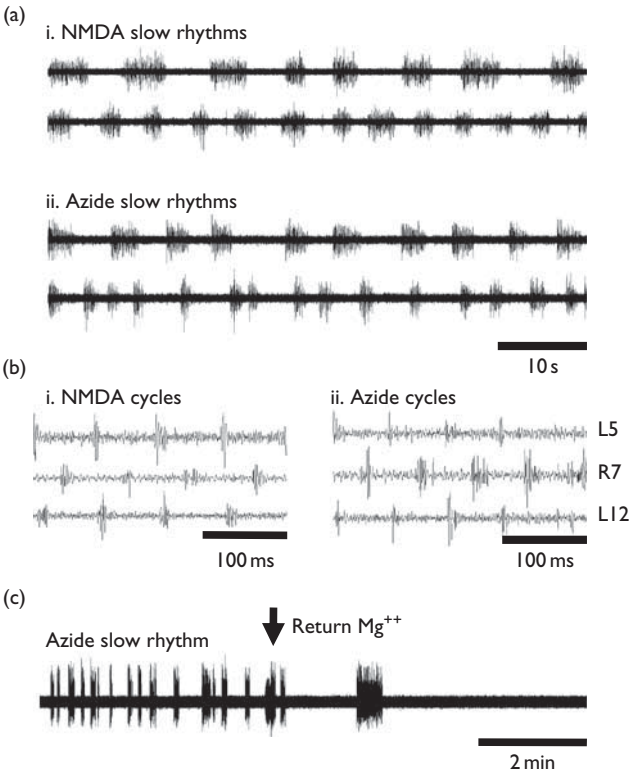


Recovery from chemical hypoxia restores swim cycle frequency, but not swim episode duration. (a) Swim cycle frequency was reduced in the presence of 5 mM azide, but it recovered to preazide values for at least 1 h after recovery. Note that cycle frequency during the slow rhythm of spontaneous swim episodes was not different from stimulated swimming, either preazide or after recovery. Data are plotted as mean ± standard error. Data are compared using one-way analysis of variance (ANOVA) followed by Holm–Sidak pairwise multiple comparisons. (b) The mean duration of swim episodes was profoundly reduced in the presence of 5 mM azide and this did not recover with superfusion of fresh saline for at least 1 h. Note that the duration of spontaneous swim episodes in the slow rhythm was not different from episode duration in azide and after recovery. Data are not normally distributed and are displayed as box plots indicating median, 25th and 75th percentiles; whiskers indicate 5th and 95th percentiles. Data compared using Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's pairwise multiple comparisons. The number of preparations is indicated in parentheses (a and b). Asterisks indicate significant difference from preazide values, daggers (in a) indicate significant difference from 5 mM azide values and a bar above data points indicates no significant difference between them.

frequency during this spontaneous swimming was the same as for preazide or recovered preparations (13.4 ± 0.6 Hz, Fig. 2a) and the episode duration was short and the same as for recovered preparations (3.6 ± 0.5 s, Fig. 2b). Slow rhythmic modulation of swim pattern generation induced by NMDA and dependent on 5-hydroxytryptamine can enhance the intensity of the motor pattern [20] and we found that the slow rhythm generated by recovery from azide-induced arrest was superficially indistinguishable from the slow rhythm induced by 50 μM of NMDA (Fig. 3).

The frequency of the azide-induced slow rhythm was 0.12 ± 0.02 Hz ($n = 10$), showing a similar variability to the NMDA-induced rhythm (Fig. 3ai and ii). Cycle frequency and other features of the motor pattern such as right/left alternation and the rostro–caudal delay were the same in both the cases and not measurably different from the preazide, stimulus-induced, motor pattern (Fig. 3bi and ii). The NMDA receptor is blocked by Mg^{++} ions and to test whether the azide-induced rhythm might be mediated by an NMDA receptor-dependent pathway, we performed azide washout using Mg^{++}-free saline and subsequently

Fig. 3



Slow rhythm of spontaneous swimming induced by recovery from chemical hypoxia is similar to the slow rhythm of swimming induced by *N*-methyl-D-aspartic acid (NMDA). (ai) Two examples of slow rhythms induced by 50 μM NMDA. (aii) Two examples of slow rhythms induced by recovery from azide pretreatment. Note the variability in the frequency of the slow rhythm in both the cases. (bi and ii) Expanded recordings of motoneuron activity from ventral roots L5 (top trace), R7 (middle trace), and L12 (bottom trace) to illustrate the right/left alternation and rostro–caudal delay of the swim motor pattern. Characteristics of the swim cycles were not different between NMDA cycles (bi) and azide cycles (bii). (c) Spontaneous swim episodes induced by recovery from azide pretreatment using Mg^{++}-free saline were inhibited by replacing Mg^{++} ions into the superfusing saline (arrow).

returned Mg^{++} to the bath. In all the three preparations, the spontaneous azide-induced rhythm was inhibited by returning Mg^{++} to the bath (Fig. 3c) suggesting that glutamate acting through NMDA receptors may mediate the rhythm-inducing effect of chemical hypoxia.

Discussion

We found that chemical hypoxia using 5 mM NaN_3 (or 1 mM KCN) reversibly abolished swimming motor pattern generation in the spinal cord of stage 42 Xenopus tadpoles. Recovery on washout was complete, with the notable exception of the duration of swim episodes evoked by tail stimulation, which remained short until the experiment was terminated (after at least 1 h recovery). Another long-lasting effect of azide-induced failure and recovery was the appearance of a slow rhythm of spontaneous episodes of swimming suggesting an increase in excitability in the spinal cord. The results we described here are similar to the results of chemical anoxia using KCN on locomotor pattern generation of neonatal rat spinal cord [21]. In that study anoxia reversibly abolished chemically or electrically evoked locomotor rhythms and this was followed by pronounced postanoxic hyperexcitability. The duration of swim episodes is partly determined by the timing of GABAergic inhibition [22]. Although GABAergic inhibition is potentiated by NO to reduce episode duration [23] and earlier we have suggested that environmental stress tunes the swim circuit through the NO/cGMP pathway [5], our current data indicate that the NO/cGMP pathway is not involved at least in the acute effects of azide. Unlike azide, KCN does not cause systemic increases of NO [17], but it had the same effect as azide in our preparations. Thus, we believe that the effects of azide we described were primarily the result of inhibition of the respiratory chain in mitochondria. The possibility remains that local cellular increases of NO, downstream from mitochondrial inhibition, may have a role to play [24]. The posthypoxic hyperexcitability we observed was in the form of a rhythmical modulation suggesting activation of glutamate NMDA receptors [20]. This is consistent with the demonstration that the rhythmical modulation is sensitive to the presence of Mg^{++} ions that block the NMDA receptor conductance at resting membrane potentials. We did not test whether the inhibition of the slow rhythm by returning Mg^{++} ions to the bath was a transient effect, but suspect that this would be true because slow rhythms were recorded in normal saline after hypoxia. Further support for the role of NMDA receptors is the observation that the NMDA antagonist MK-801 prevents the effect of NaN_3 on cell viability [13,15] and on intracellular calcium concentration in rat primary cortical neurons [25] indicating a key role for glutamate acting through NMDA receptors in mediating the effects of azide in neurons.

Conclusion

We conclude that hypoxia, which is an environmental hazard for tadpoles, has long-term effects on the neural circuits underlying swimming through a coordinated modulation of several transmitter pathways. We propose that these changes are adaptive. Swim episodes are kept short to conserve energy, but the activation of intermittent swimming would be appropriate to remove the animal from the hypoxic environment.

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