Hydrogen sulfide mimics hypoxic response at the respiratory cranial nerves of the aquatic anuran *Xenopus laevis*.

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Abstract:

Previous studies have shown that in vitro brainstems from post-metamorphic bullfrogs exposed to hypoxia causes a cessation of respiratory-related nerve activity (fictive breathing). The mechanism underlying the cessation of fictive breathing is unknown, although blocking K_{ATP} channels and adenosine receptors attenuates this response. In this study we tested the hypothesis that hydrogen sulfide (H_2S) contributes to the hypoxic-induced cessation of fictive breathing in the isolated amphibian brainstem. Our hypothesis is based on findings that H_2S contributes to hypoxia-induced reflexes in vascular tissue and may be an important signaling molecule for oxygen-sensing. Our goal in this study was two-fold: 1) to examine the effect of hypoxia on the isolated brainstem from an aquatic African clawed frog (*Xenopus laevis*) and compare the breathing patterns and responses with previous data from a semi-aquatic bullfrog brainstem, and 2) to test whether application of H_2S to the post-metamorphic frog brainstem mimics the effects of hypoxia. Brainstems were isolated and removed from newly-metamorphosed frogs under Tricaine anesthesia and placed in a recording chamber at room temperature (22-24 °C). Brainstems were superfused continuously with an artificial CSF (aCSF) bubbled with 98% O_2/2% CO_2 (control) or hypoxic aCSF (98% N_2/2% CO_2) for up to 4 hours. Fictive breathing was recorded with suction electrodes applied to cranial nerves (V & X) that normally innervate respiratory muscles. In a separate group of animals, brainstems were given various concentrations of sodium sulfide ($Na_2S \cdot 9H_2O$ - a H_2S donor) in control aCSF. Brainstem hypoxia exposure in *X. laevis* showed a similar cessation of fictive breathing as found with bullfrogs. Sodium sulfide decreased the amplitude of fictive lung bursts at 200 µM and caused cessation of fictive breathing at 400 µM. The results indicate that hypoxia causes cessation of fictive breathing in an aquatic amphibian brainstem preparation and that H_2S mimics the response to hypoxia. Hydrogen sulfide may be a key molecule for the hypoxia-induced respiratory depression in the amphibian brainstem.

Introduction:

- Previous *in vitro* studies in adult bullfrogs (terrestrial anuran) show that the motor cranial nerves responsible for the major respiratory activity, the trigeminal (V) and vagus (X) nerves, cease firing under hypoxic aCSF conditions (Winmill, Chen & Hedrick, 2005).
- However, owing to the fact that *X. laevis* are aquatic throughout their entire life cycle and that subtle differences exist in their respiratory mechanism when compared to rana and lithobates, it is ill-advised to assume rana and lithobates data can be fully extrapolated to xenopus (Gargaglioni & Milsom, 2007).
- When no stress stimulus is present *X. laevis* may either display a long term series of breaths at the surface or rapidly breathe and dive (Boutilier, 1984).
- Hydrogen sulfide has been implicated as a hypoxia signal in several different tissues.

Methods:

Animals and brainstem removal: Frogs were purchased from Xenopus Express and shipped overnight. They were housed in large tanks on a recirculation system with the water maintained at 20-22°C and were fed twice a week. Prior to brainstem removal the animals were fully anesthetized with a 0.30% solution of Tricaine. Brainstems were removed and mounted in a recording chamber.

Nerve recordings: Suction electrodes were attached to two cranial nerves for each brainstem preparation (typically one on each side and either vagus or trigeminal). The signals were passed through a differential amplifier and an integrator before being recorded through a PowerLab module with LabChart.

Control measurements: Recordings were taken continuously for 4-6 hours while the brainstem was being superfused with normoxic (98% O_2 / 2% CO_2) artificial cerebrospinal fluid (aCSF).

Hypoxic measurements: A baseline recording was taken for one hour with normoxic aCSF followed by a one hour hypoxic measurement switching to hypoxic (98% N_2 / 2% CO_2) aCSF. Finally the brainstem was returned to a normoxic aCSF flow for a final hour.

Sodium sulfide (Hydrogen sulfide) measurements: Similar to the hypoxic measurements, one hour baseline and post-exposure measurements were taken. A one hour measurement was taken with various concentrations (50, 100, 200, 400 and 600) µM of nonahydrate sodium sulfide (a hydrogen sulfide donor), normoxic aCSF.

Data analysis and figures: Statistical analysis and graphs were constructed using GraphPad Prism. Nerve traces were taken from LabChart ZoomView.

Results:

A. Exposure of the brainstem and cranial nerves to hypoxic aCSF caused a decrease of both average frequency of nerve impulses from 3.42 ± 0.13 peaks per minute (mean ± SEM) to 0.10 ± 0.04 peaks per minute and average relative amplitude from 0.249 ± 0.04 to 0.054 ± 0.01. After the one hour hypoxia exposure all four brainstems recovered activity and returned to approximate pre-hypoxia frequency: 5.99 ± 1.62 peaks per minute and amplitude 0.289 ± 0.07. Using a repeated measure ANOVA both the frequency and amplitude were shown to have significant changes ($p=0.0048$ and $p=0.0043$ respectively) and Newman-Keuls multiple comparison test showed that hypoxic aCSF amplitude and frequency were significantly different from both the pre and post hypoxic recordings ($p<0.05$). Pre and post hypoxic levels were not shown to be significantly different (Figure 1).

B. Hydrogen sulfide caused a noticeable decrease in the number of detectable fictive breaths starting at 100 µM concentrations of Na_2S with a significant loss of most detectable signals starting at 400 µM. A possible downward trend exists in the amplitudes for the signals that do occur. These comparisons were made disregarding the initial burst of high frequency activity (see discussion) when it did occur. Statistical significance can not currently be determined due to a large variance and limited sample sizes (n=2 or 3 animals per concentration) (Figure 2).

Figures:

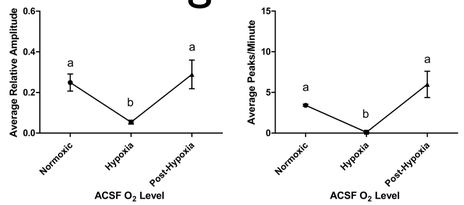

Figure 1: Hypoxic aCSF decreases both frequency and average relative amplitude in *X laevis* trigeminal and vagus nerves. A post-hopoxic recovery then occurred that was at least equal to pre-hypoxic baseline readings ($p=0.42$ by paired T-test).

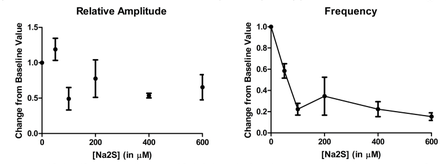

Figure 2: Increasing hydrogen sulfide (from sodium sulfide) levels lead to a steady decrease in fictive breath frequency and an overall decrease in amplitude at 400 and 600 µM concentrations even in the presence of normoxic aCSF.

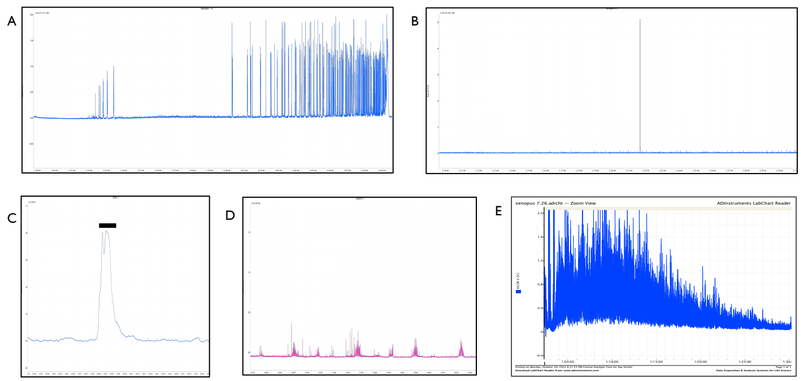

Figure 3: Nerve traces. a) One cycle from control recordings (20 minute duration). b) One twenty minute sample of a hypoxic aCSF recording. c) One fictive breath, reference bar is approximately 2 seconds. d) Representative nerve trace of the semi-aquatic anuran *Lithobates pipiens* (10 minute duration). e) Occasional and so far believed to be random response of high frequency nerve impulses to hydrogen sulfide.

Discussion:

A cyclic pattern of fictive breaths was present under normoxic aCSF condition in all but one recording. Fictive breaths occurred nearly continuously for 15.8 ± 6.75 minutes (mean ± SD) and is followed by a period of fictive apnea for 8.3 ± 5.65 minutes where no fictive breathing signals were found. To our knowledge this long duration pattern of respiratory nerve signals is not present in terrestrial amphibians and suggests a possible link to surfacing and diving behavior (Figure 3a,c,d).

The *in vitro* cranial nerve response to hypoxic aCSF in *X. laevis* was shown to be similar to the cessation of fictive breaths found in previous studies of terrestrial amphibians (Figure 3b).

It was noted that after hypoxic aCSF exposure that the frequency of fictive breaths tends to increase when compared to the baseline. The amplitude of signals also increased slightly, but this appears to be to a lesser degree. This may represent an adaptive plasticity in response to hypoxia; however, a statistically significant relationship was not found with our current sample size.

In general, it took between 8 and 15 minutes to affect the desired change in the brainstem when a switch in aCSF composition was made. We believe, though have not yet tested, that this extended delay is primarily due to the time for the composition in the recording chamber to be fully altered.

A pattern of extremely rapid nerve activity which decreases from high amplitude towards minimum amplitude was present in some of the hydrogen sulfide recordings. This pattern appears to be a random occurrence and has, to date, appeared in 100, 400 and 600 µM concentrations and can last as long as 30 minutes. It is present in 5 out of 24 nerve recordings we have made under sodium sulfide conditions and has yet to appear in two nerve recordings taken from the same brainstem (Figure 3e).

Future Directions:

Compare these responses with pre-metamorphic *X. laevis*.

Investigate the possibility of plasticity of cranial nerves in response to a hypoxic stimulus.

Further examine the role of hydrogen sulfide in the nervous system hypoxia signal and response pathway with H_2S enzyme inhibitors.

References:

Boutilier, R.G. (1984). Characterization of the intermittent breathing pattern in *Xenopus laevis*. J. Exp. Biol. 110: 291-309.

Gargaglioni, L.H., and Milsom, W.K. (2007). Control of breathing in anuran amphibians. Comparative biochemistry and physiology part a: Molecular & integrative physiology. 147(3): 665-684.

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