

Slow death in the leopard frog *Rana pipiens*: neurotransmitters and anoxia tolerance

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Summary

While frogs such as *Rana temporaria* are known to withstand 4–5 h anoxia at room temperature, little is known about the neurological adaptations that permit this. Previous research has shown that changes in neuroactive compounds such as glutamate and dopamine in anoxia-sensitive (mammalian) brains follow a strikingly different pattern than is observed in truly anoxia-tolerant vertebrates such as the freshwater turtle. The present study measured changes in the levels of whole brain and extracellular amino acids, and extracellular dopamine, in the normoxic and 3–4 h anoxic frog *Rana pipiens*, in order to determine whether their neurotransmitter responses resemble the anoxia-vulnerable or anoxia-tolerant response. Increases in whole brain serine, glycine, alanine and GABA levels were similar to those seen in anoxia-tolerant species, although the levels of glutamine, taurine and glutamate did not increase as occurs in true

facultative anaerobes. Extracellular levels of aspartate, taurine and GABA also increased significantly, while glutamate levels decreased. The maintenance of low extracellular glutamate was the most significant difference between the frog and the anoxic/ischemic mammalian brain, although aspartate did increase 215% over a 4 h period of anoxia. A 12-fold increase in extracellular dopamine levels during anoxia was the biggest contrast between anoxia-tolerant vertebrates and *R. pipiens*. The frog could thus be an interesting model in which to examine the mechanisms of dopamine failure in early anoxia, which occurs rapidly in the mammal but over a period of hours in the ‘slow death’ of the anoxic frog brain.

Key words: anoxia, frog, *Rana pipiens*, excitatory amino acid, dopamine, slow death.

Introduction

The effects of anoxia/ischemia on the mammalian brain, and the mechanisms that promote survival or neuronal failure, have been extensively studied owing to their great significance in human pathophysiology. In brief, the mammalian brain loses ATP within minutes of oxygen deprivation, with a subsequent failure of ATP-dependent ion exchangers, the loss of ionic gradients, and membrane depolarization. Depolarization then results in a cytotoxic increase in intracellular Ca^{2+} concentration, the uncontrolled release of excitatory neurotransmitters to neurotoxic levels, and subsequent neuronal death (for a review, see Lutz et al., 2003). Once temperature differences are taken into account, this scenario is in fact the characteristic response of nearly all vertebrate brains, from fish to mammals (Lutz et al., 2003). A few species, however, including turtles in the genera *Trachemys* and *Chrysemys* and fish in the genus *Carassius*, can survive anoxia for days at 25°C and months at temperatures below 10°C (Lutz et al., 2003). These animals are able to greatly reduce brain metabolic rates to a level where energy costs are matched by anaerobic energy production; ATP levels are thus maintained and anoxic depolarization is avoided. Specific adaptations by

which the brains of the anoxia-tolerant organisms survive include a reduction in membrane ion permeability (channel arrest) (Bickler et al., 2002), the release of inhibitory neurotransmitters such as GABA and alanine (Nilsson and Lutz, 1991), and protection against the uncontrolled release of such excitotoxic compounds as glutamate and dopamine (Milton and Lutz, 1998; Milton et al., 2002).

Some frog species (*Rana temporaria*, *Rana pipiens*) demonstrate an intermediate anoxia tolerance, their brains able to survive 4–5 h without oxygen at room temperature (Knickerbocker and Lutz, 2001; Lutz and Reiners, 1997) and at least 30 h without oxygen at 5°C (Hermes-Lima and Storey, 1996). This tolerance to anoxia appears to be accomplished through an overall metabolic depression, primarily via hypoperfusion of the skeletal muscle (Donohoe and Boutilier, 1998; Donohoe et al., 1998), which allows the frog to maintain ATP levels in certain organ systems. However, unlike truly anoxia-tolerant vertebrates, the frog brain does not defend ATP levels, and when energy stores reach a critical low, (approximately 35% of normoxic levels), ion homeostatic mechanisms are compromised and extracellular K^+ levels

begin to increase (Knickerbocker and Lutz, 2001). Extracellular $[K^+]$ reaches a critical threshold within an additional 1–2 h, after which there is a rapid K^+ efflux, indicating anoxic depolarization (Knickerbocker and Lutz, 2001), accompanied by a massive loss of neurotransmitters (Lutz and Reiners, 1997). This pattern of ATP loss, ion leakage and depolarization in response to anoxia in the frog brain is the same as that observed in the anoxic/ischemic mammalian brain, but critically low ATP levels are reached in only a few minutes in the mammalian brain, *versus* approximately 3 h in the frog brain (Lutz and Reiners, 1997; Knickerbocker and Lutz, 2001). The enhanced anoxia tolerance of the frog brain thus appears to be a case of 'slow death', with the same sequence of events that occur catastrophically in the mammalian brain but on a greatly extended time scale. This could make the frog brain a model of particular interest for investigating the processes of anoxic/ischemic failure.

There is, however, a lack of basic information on adaptations exhibited by the frog that enable them to extend anoxic death over a period of hours. The present study examines the effects of anoxia on tissue and extracellular neurotransmitter levels, as anoxia is known to cause very substantial changes in these compounds in the vertebrate brain. Anoxia-tolerant species such as the crucian carp and freshwater turtle tend to exhibit similar patterns of changes in these compounds, i.e. increases in whole brain and extracellular levels of inhibitory compounds such as GABA, glycine and taurine, and decreases in the levels of excitatory amino acids such as glutamate and glutamine (Nilsson et al., 1990). By contrast, anoxia-intolerant animals (brown anole, neonatal and adult rat) demonstrate increases only in tissue GABA and/or alanine levels, while whole brain levels of glutamate and glutamine may even increase (Nilsson et al., 1991; Lutz et al., 1994; Erecinska et al., 1984); neurotransmitters are then released indiscriminately upon depolarization (Lutz et al., 2003).

There is little known about the effect of anoxia on neurotransmitter release in the frog brain. Lutz and Reiners (1997) found a massive increase in extracellular glutamate and GABA levels upon anoxic depolarization in the frog brain, but numerous other neuroactive compounds have not yet been investigated. These include neuroprotective amino acids such as taurine and alanine as well as neurotoxic compounds such as dopamine. In the turtle, extracellular glutamate and dopamine are maintained at basal levels during extended anoxia (Nilsson and Lutz, 1991; Milton and Lutz, 1998), while levels of inhibitory neuroactive compounds such as GABA and glycine increase (Nilsson and Lutz, 1991). By contrast, extracellular neurotransmitter levels increase indiscriminately in anoxia-intolerant animals: dopamine, aspartate, glutamate, GABA and taurine all increase in the hypoxic or anoxic neonatal rat and adult rat brain (Huang et al., 1994; Perez-Pinzon et al., 1993; Young et al., 1993), while glutamate, aspartate, GABA, and taurine are all released from hippocampal slices exposed to hypoxia, chemical ischemia or hydrogen peroxide (Saransaari and Oja, 1998).

Dopamine is of particular interest because this compound is

neurotoxic at high levels, but unlike other excitotoxins such as glutamate, dopamine levels increase in the mammalian extracellular compartment well before anoxic or ischemic depolarization (Huang et al., 1994; Globus et al., 1988). By contrast, extracellular dopamine levels do not increase in the anoxic turtle brain due to both decreased release (S. L. Milton and P. L. Lutz, manuscript in preparation) and continued reuptake (Milton and Lutz, 1998).

The present study measured the effects of anoxia on levels of neurotransmitters in the whole brain and extracellular space in the frog *Rana pipiens*, in order to determine whether neurotransmitter responses during 'slow death' more closely resemble the anoxia-vulnerable (mammalian) or anoxia-tolerant (turtle, carp) response to anoxia.

Materials and methods

All experiments were approved by the Florida Atlantic University Institutional Animal Care and Use Committee. All experiments were performed on northern leopard frogs *Rana pipiens* Shreber 1782 weighing 30–55 g, obtained from commercial suppliers (Charles D. Sullivan Co., Nashville, TN, USA). Animals were housed at room temperature and subjected to a 12 h:12 h light:dark cycle; frogs had constant access to freshwater and were fed crickets *ad libitum* until 5 days prior to experiments.

Tissue sampling: whole brain amino acids

All experiments were performed at room temperature ($25 \pm 1^\circ\text{C}$). Control animals were removed from their holding tank and decapitated. The brains were dissected out in less than 1 min and frozen in liquid nitrogen. Anoxic animals were held in a sealed polyethylene chamber under positive-flow nitrogen (99.99% nitrogen, County Welding, Pompano Beach, FL, USA) for 1, 2 or 4 h anoxic exposure. At these time points, the frogs were removed from the anoxic chamber, and their brains removed and treated as above. Samples were held at -80°C .

While still frozen, brain sections were weighed and homogenized in 20 volumes of 4% (w:v) ice-cold perchloric acid buffered with 0.2% EDTA and 0.05% sodium bisulfate, using a Sorval Teflon-glass homogenizer. The homogenate was cold-centrifuged at 15 000 g for 5 min and the supernatant stored at -20°C until analysis.

Tissue sampling: microdialysis

The frogs were placed in a 500 ml sealed polyethylene box containing saline in which was dissolved the anesthetic tricaine methanesulfonate, MS-222 ($250\text{--}300\text{ g l}^{-1}$) buffered to a pH of 7.4. Until surgery the chamber was continuously aerated with 100% O_2 . After a surgical plane was achieved (as indicated by lack of ocular reflex), an area ($4\text{ mm} \times 5\text{ mm}$) of the skull directly over the cerebral hemispheres was removed; a small incision through the dura mater exposed the cerebral hemispheres. A stereotaxic instrument and guide were used to insert a CMA 12 microdialysis probe (1 mm membrane length, Bioanalytical Systems, Inc., Acton, MA, USA) into the

hemispheres (2 mm depth from the cerebral surface). After a 1 h stabilization period during which no sampling occurred, the probe was perfused with frog ACSF solution (100 mmol l⁻¹ NaCl, 3.5 mmol l⁻¹ KCl, 26 mmol l⁻¹ NaHCO₃, 1.25 mmol l⁻¹ NaH₂PO₄, 2.0 mmol l⁻¹ CaCl₂, 2.0 mmol l⁻¹ MgSO₄ and 2.0 mmol l⁻¹ glucose, pH 7.4) at 2.0 µl min⁻¹ with a CMA/100 microdialysis pump (Carnegie Medecin, Solna, Sweden). Anoxia was induced in experimental groups by changing the aerating gas to 100% nitrogen; animals remained anoxic for 4 h. Control animals remained in oxygen-aerated chambers for 4 h; all experiments were performed at 15–18°C. After all experiments, Methylene Blue was injected through the microdialysis probe to identify the probe location. Data were utilized only from those animals in which the probe location was verified. Probe recovery (amino acids and dopamine) was determined from known standards *in vitro* (Huang et al., 1994); mean recoveries were 20–23%.

Amino acid analysis

The amounts of amino acids present in the tissue supernatants obtained after centrifugation and in dialysate samples were quantified by reversed-phase high-performance liquid chromatography (HPLC) with fluorescent detection. The HPLC system consisted of a Knauer HPLC pump 64 (Sonntek, Inc., Upper Saddle River, NJ, USA) and E-lab gradient controller (OMS Tech, Miami, FL, USA), a reversed-phase column (Adsorbosphere OPA 5u, 150 mm × 4.6 mm, Alltech, Deerfield, IL, USA), and an RF-535 fluorescence detector (Shimadzu, Kyoto, Japan). For microdialysis samples, 20 µl dialysate was mixed with 30 µl complete *o*-phthalaldehyde reagent solution (Sigma) at 25°C; after exactly 1 min, 30 µl was injected onto the HPLC column. Data are presented as percentage of control due to baseline variability between animals.

Tissue amino acid concentrations are given as µmol g⁻¹ wet mass brain tissue. All values are expressed as mean ± S.E.M. Statistical significance of changes for all data was determined using one-way analysis of variance (ANOVA/Student's *t*-test) utilizing the SAS/JMP statistical package (Cary, NC, USA); in cases of unequal variance, data were treated non-parametrically (Kruskal–Wallis test for unequal variances). *P* < 0.05 was considered to be statistically significant. Normoxia (control) was calculated as the sample mean for the hour immediately preceding anoxic exposure, 1 h of anoxia as the mean of samples from time zero (start of anoxia) to 1 h of anoxia, and 4 h of anoxia as the mean of samples collected from 3 h to 4 h of anoxic exposure.

Dopamine analysis

Samples were analyzed for monoamine content using HPLC with electrochemical detection, as adapted from Nilsson (1990). A 20 µl sample of dialysate was injected into a 510 HPLC pump (Waters, Milford, MA, USA; flow rate 1.3 ml min⁻¹). The mobile phase consisted of 100 mmol l⁻¹ NaH₂PO₄, 9% (v/v) methanol, 0.63 mmol l⁻¹ sodium octyl sulfate and 0.2 mmol l⁻¹ EDTA, pH 3.6. Samples were

separated on a Catecholamine C18 column (3 µm, 100 mm × 4.6 mm; Alltech) and detected by an LC-3 electrochemical detector with a glassy carbon working electrode set at +750 mV. Concentrations were determined by integrating the area under the peak compared to known standards. Integrations were performed using the Dynamax MacIntegrator II Integrator and software (Rainin Instrument Corp., Woburn, MA, USA).

Results

The frog brain exhibits some of the adaptations that are so prominent in true facultative anaerobes like the freshwater turtle, but the overall pattern of changes in neuroactive compounds more closely resembles those in the anoxia-intolerant mammalian brain.

Whole brain amino acids

Amino acid concentrations in the frog forebrain resemble the levels observed in other vertebrate brains (e.g. neonate rat, Lutz et al., 1994; brown anole and *Trachemys*, Nilsson et al., 1991) with most in the range of 1–3 µmol g⁻¹; glutamine and glutamate predominate with concentrations of 13.68±1.964 and 9.00±0.805 µmol g⁻¹, respectively (Fig. 1). Changes in whole brain amino acid levels over a 4 h period of anoxia appear to be intermediate on the anoxia-tolerant to anoxia-intolerant scale. Changes that were similar to those seen in anoxia-tolerant species included increases in the concentrations of serine, glycine, alanine and GABA. Remarkably, no significant changes occurred over the first hour of anoxia, though a slight increase in alanine was detected. By 2 h of anoxia, alanine levels had increased significantly to 220% of control while GABA increased by 40%. By 4 h of anoxia, alanine had increased further to 330% of control levels, with no additional changes in GABA. Glycine and serine levels were 193% and 225% of control, respectively, after 4 h of

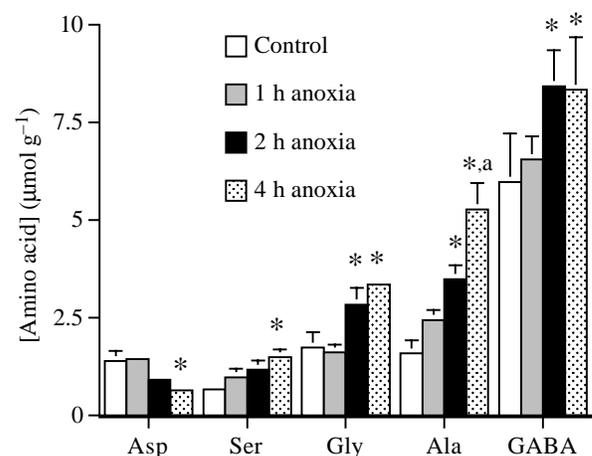


Fig. 1. Forebrain amino acid levels in the 1–4 h anoxic frog *Rana pipiens*; *N* = 5 per time point. For details of sample times, see text. *Significant difference from control, ^a4 h time point significantly different from 2 h, *P* < 0.05.

anoxia. Aspartate levels decreased by 54% over 4 h of anoxia; changes in whole brain aspartate levels in response to anoxia have not been previously reported.

Whole brain amino acid levels that were not altered by 4 h anoxia included glutamine, taurine and glutamate (Fig. 2). Increases in whole brain taurine and decreases in glutamate and glutamine have been reported in anoxia-tolerant animals, but do not change in anoxia-sensitive species.

Extracellular amino acids

Extracellular levels of aspartate, taurine and GABA increased in a statistically significant manner, while glutamate decreased (Fig. 3). Extracellular aspartate levels increased to $145 \pm 6\%$ of control by 1 h of anoxia; this increase was significant by 4 h of anoxia, when mean levels were $215 \pm 41\%$ of control. Taurine levels increased by $24 \pm 5.5\%$ over 4 h of anoxia, while GABA levels increased by 45% after 1 h of anoxia and increased significantly to $302 \pm 78\%$ of control by

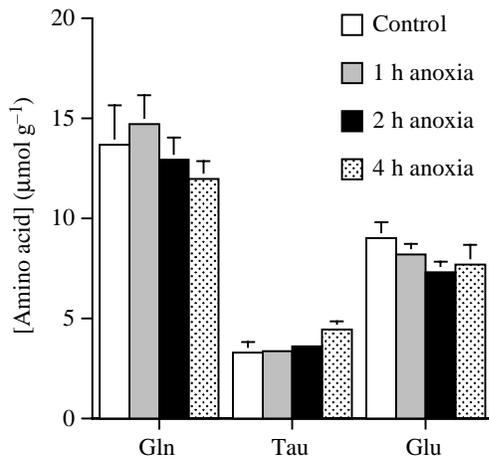


Fig. 2. Whole brain (forebrain) amino acids in the frog *Rana pipiens* unchanged over 1–4 h of anoxia; $N=5$ per time point.

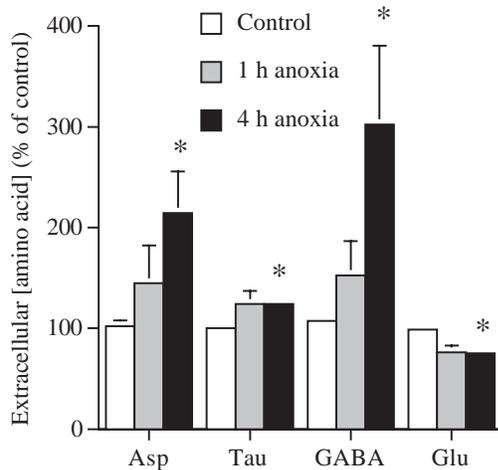


Fig. 3. Changes in forebrain extracellular amino acids from control levels in 1 h and 4 h anoxic frogs *Rana pipiens*; $N=5-6$ per time point. *Significant difference from control, $P<0.05$.

4 h. On the other hand, unlike anoxia-intolerant organisms, glutamate levels did not increase, and in fact decreased by approximately 25% over a 4 h period of anoxia. Extracellular amino acid levels that did not change during anoxia included serine, glutamine, glycine and alanine (Fig. 4).

Dopamine

The largest contrast between anoxia-tolerant vertebrates and *R. pipiens*, however, was in extracellular dopamine (DA). Mean DA levels prior to anoxic exposure (17.0 ± 2.49 ng ml⁻¹) did not significantly differ from control levels (12.9 ± 1.6 ng ml⁻¹), nor did control DA levels increase over a 3 h period of normoxia. Extracellular DA levels began to rise immediately upon anoxic exposure, however, and were significantly different from pre-anoxic levels by 2 h of anoxia. By 4 h of anoxia, dopamine had increased 12-fold over normoxic control levels (Fig. 5).

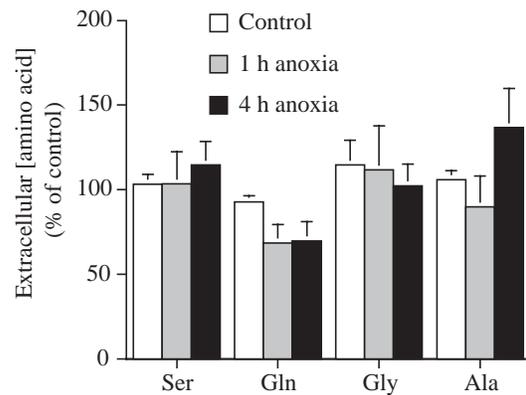


Fig. 4. Forebrain levels of extracellular amino acids unchanged over a 4 h period of anoxia in the frog *Rana pipiens*; $N=5-6$ per time point.

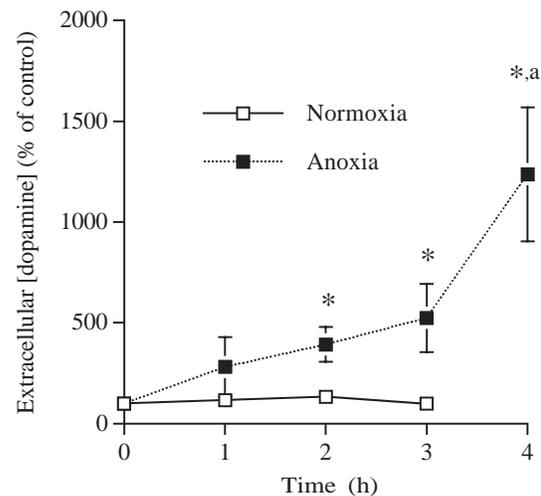


Fig. 5. Forebrain extracellular [dopamine] in the normoxic vs. 4 h anoxic frog *Rana pipiens*; $N=6$ for normoxic group, $N=5$ for anoxic group. *Significantly different from control, ^asignificantly different from previous time point.

Discussion

The frog brain, while clearly able to survive a 4 h period of anoxia at room temperature, exhibits only some of the adaptations that are so prominent in true facultative anaerobes like the freshwater turtle, and overall more closely resembles the anoxia-intolerant mammalian brain. One interesting facet of its anoxia tolerance is that no changes in whole brain neurotransmitter levels occur during the first hour of anoxia, despite the fact that ATP levels begin to fall during this period, though extracellular changes do occur. By contrast, nearly all neurotransmitters change significantly (up or down) by 1 h of anoxia in the anoxia-tolerant turtle. This is possibly a reflection of the different strategies for coping with the anoxic stress. Frogs are known to be extremely hypoxia tolerant (Donohoe et al., 1998), relying on hypometabolism and energy redistribution to decrease ATP demand when energy supply is compromised (West and Boutilier, 1998). The initial hour may then represent a period of severe hypoxia, in which oxygen stores are being depleted and ATP levels decrease but have not yet reached critical levels. The frog may thus be maintaining an internal *status quo* while releasing inhibitory neurotransmitters into the extracellular space. (It may also simply be that changes are beginning to occur intracellularly but not at detectable levels.) The turtle, on the other hand, appears to use the first hour of anoxia to actively shut down metabolic processes in preparation for long-term anoxia. The first hour of anoxia is marked by temporary increases in extracellular adenosine levels and blood flow (Hylland et al., 1994), the decreased release of dopamine and glutamate (S. L. Milton and P. L. Lutz, manuscript in preparation; Milton et al., 2002) and the active downregulation of ion channels (Bickler et al., 2002).

By 2 h of anoxia in the frog brain, however, significant changes have occurred in both intra- and extracellular neurotransmitter levels.

Alanine

Since alanine serves as an end product of glycolysis (Hochachka and Somero, 2002), the increase in alanine and its continued rise indicates an early and sustained activation of glycolysis in the anoxic frog brain. Besides being an alternative anaerobic end product to lactate, alanine may also mediate a decrease in glycolytic rate by inhibiting pyruvate kinase, such that even small amounts may act as an important regulator of the hypometabolic state (Hochachka, 1980). In the turtle *Trachemys scripta*, alanine concentrations between 0.16 mmol l⁻¹ (heart and liver) and 1.08 mmol l⁻¹ (white muscle) decreased pyruvate kinase activity by 50% (Brooks and Storey, 1989). An increase in brain [alanine] during anoxia/ischemia appears to be a common vertebrate response, having been found in the anoxic turtle (Nilsson and Lutz, 1991) crucian carp (Hylland and Nilsson, 1999) and neonate rat (Lutz et al., 1994) as well as the ischemic adult rat brain (Erecinska et al., 1984; Young et al., 1993). It has been suggested that increasing alanine levels may even be a preferred indicator of severe hypoxia, as lactate is a reliable indicator of only mild

hypoxia (Ben-Yoseph et al., 1993). However, the increase in alanine concentration in true facultative anaerobes (approximately tenfold in the carp and turtle) is far greater than occurs in the anoxia/ischemic mammalian brain (~60% in the neonate rat). The threefold increase in alanine concentration in the frog brain over a 4 h period of anoxia, then, is greater than observed in anoxia-sensitive species, if not of the magnitude seen in the carp or turtle. Interestingly, extracellular alanine levels did not increase despite the threefold increase in intracellular levels; it may be that intracellular alanine is conserved to serve as a brain energy substrate when oxygen supply is restored; extracellular alanine levels may be biologically less important than intracellular changes, as it is unlikely that alanine acts as a neurotransmitter (Nilsson et al., 1990).

GABA and glycine

The increase in both whole brain and extracellular GABA levels seen in the anoxic frog brain may also be common to anoxic/ischemic vertebrate brains, having been found in anoxic/ischemic mammals as well as the crucian carp and turtle (Nilsson and Lutz, 1990; Nilsson, 1990). GABA is a well-established inhibitory neurotransmitter in the vertebrate brain that increases chloride influx into the neuron and reduces hypoxic/ischemic damage, in part by decreasing glutamate release (Johns et al., 2000). Extracellular increases in GABA levels, as in the anoxic turtle, appear to be due to a sustained release from intracellular sources rather than mere overflow. The 41% increase in total GABA concentration in the frog echoes the 45–60% increase in turtle whole brain GABA concentration, but extracellular levels increase to a much greater degree (to 300% of control by 4 h) than intracellular levels. This increase is still quite small compared to that in the turtle (which shows a 90-fold increase over 6 h; Nilsson and Lutz, 1991), thus the utility of GABA as an inhibitory neurotransmitter in the frog may be a case of 'too little, too late'. As in the anoxic mammal, the greatest increases in extracellular GABA concentration occur after depolarization (Lutz and Reiners, 1997), when the response is pathological rather than adaptive, though even at that point high concentrations of inhibitory compounds released concomitantly with excitotoxic ones may constitute a protective mechanism against excess excitatory amino acids (Saransaari and Oja, 1998). Such protective mechanisms simply appear to be expressed earlier and more robustly in anoxia-tolerant organisms like the turtle.

Like GABA, glycine is a well-described inhibitory neurotransmitter in the brainstem and spinal cord of mammals, where it opens post-synaptic glycine-gated chloride channels (Wheeler et al., 1999), and may also lead to the internalization of *N*-methyl-D-aspartate (NMDA) receptors (Nong et al., 2003), though it is also excitatory in the higher brain as an allosteric activator of glutamate (NMDA) receptors (Jones and Szatkowski, 1995) and is thus likely to contribute to ischemic neuronal damage (Saransaari and Oja, 2001). The increase in glycine concentration seen after a 4 h period of anoxia in the

frog brain, however, was not reflected in extracellular changes, thus it is unlikely to have any significant neuroactive effect. In anoxia-tolerant species an increase in both intracellular and extracellular brain glycine levels is common (Nilsson et al., 1991; Nilsson and Lutz, 1991), while extracellular increases in the mammalian brain are common under anoxic or ischemic conditions (Jones and Szatkowski, 1995; Tan et al., 1996; Li et al., 1999; Saransaari and Oja, 2001) and upon depolarization (Fabricius et al., 1993), and are also seen in anoxia-intolerant ectotherms (Hylland et al., 1995).

Taurine

As with changes in GABA and alanine, an increase in extracellular taurine levels appears to be a common vertebrate response to anoxia, though unlike in the anoxia-tolerant turtle, tissue levels did not increase in *R. pipiens*, nor do they increase in the rat or anole. The increase observed here in the anoxic frog is similar to those seen in other animals: extracellular taurine levels increased approximately 34% over 4 h anoxia in the crucian carp (Hylland and Nilsson, 1999), while 4 h anoxia in the frog resulted in a 24% increase. Taurine is thought to play a variety of protective roles in the hypoxic/ischemic brain; hypoxia, anoxia, and ischemia all increase taurine release in mammals (al-Bekairi, 1989; Saransaari and Oja, 1998).

Aspartate, glutamate and glutamine

No significant changes in brain glutamate or glutamine concentrations or in extracellular levels was observed, while aspartate showed a significant decrease by 4 h in the whole brain coupled to a significant increase in the extracellular space. This lack of change in tissue levels is similar to mammals and other anoxia-intolerant animals during anoxia/ischemia (Wallin et al., 2000); both glutamate and glutamine levels decline in the brain tissue of *T. scripta* (Nilsson et al., 1990) as well as in other hypoxia-tolerant animals like the shore crab *Carcinus maenas* (Nilsson and Winberg, 1993). The lack of extracellular increase, however, is noteworthy, as pathological increases in extracellular glutamate levels are thought to play a central role in hypoxic/ischemic neuronal degeneration and reperfusion injuries (Globus et al., 1988; Choi, 1992). Extracellular glutamate levels may increase as much as 37-fold in the ischemic rat brain (Goda et al., 1998), while Young et al. (1993) found that levels in anoxic rat brain slices more than doubled in 10 min. Excess glutamate then activates ionotropic and metabotropic receptors, triggering a cascade of events resulting in neuronal death. In the frog, glutamate is apparently released only after depolarization (Lutz and Reiners, 1997), which suggests that glutamate is either not initially released in the anoxic frog or that uptake mechanisms are still active even as ATP is depleted. Such continued release and reuptake, albeit at reduced levels, was recently shown to occur in the turtle (Milton et al., 2002). It may also be that an increase in extracellular glutamate levels is in part prevented by the continued conversion of glutamate into GABA *via* glutamate decarboxylase; a slight but statistically insignificant (14%)

decrease in tissue glutamate levels did occur in the anoxic frog brain. In the frog, however, these processes eventually fail and a massive, mammalian-like increase in extracellular glutamate levels occurs; it would be interesting to determine if this is a cause or an effect of depolarization.

The fall in tissue aspartate concentration, corresponding to a rise in the extracellular compartment, suggests there may be a shift from the intra- to the extracellular compartment over a 4 h period of anoxia. Extracellular aspartate levels increase in anoxia-intolerant animals, including the rat, during cortical spreading depression and upon anoxic depolarization (Fabricius et al., 1993) and in the anoxic rainbow trout (Hylland et al., 1995). Aspartate is considered to be neurotoxic when released under cell-damaging conditions, compounding the effects of other excitatory amino acids such as glutamate (Saransaari and Oja, 1998; Zeitlow et al., 2002).

Dopamine

The most striking difference we found, however, between anoxia-tolerant animals and the frog was in the frog's lack of ability to regulate extracellular dopamine levels. The uncontrolled release of dopamine (DA) into the extracellular space of the mammalian brain has been identified as one major cause of hypoxic/ischemic brain damage, and unlike the widespread release of excitatory amino acids, which occurs only upon brain depolarization (Rothman and Olney, 1986; Baker et al., 1991), DA release is seen well before high energy stores are fully depleted (Huang et al., 1994). While severe hypoxia or ischemia can increase extracellular levels as much as 500-fold (Globus et al., 1987, 1988), even mild hypoxia (11% cortical oxygen levels) can cause neuronal damage, with DA increases as high as 200% (Huang et al., 1994). In hypoxia-vulnerable mammals, this DA increase is highly toxic and causes neuronal apoptosis in the striatum (McLaughlin et al., 1998) and in cell cultures (Zeitlow et al., 2002). Extracellular DA may contribute to neuronal damage by altering cerebral blood flow and glucose metabolism (Globus et al., 1987), through the production of reactive oxygen species (Remblier et al., 1999), by modulating the release of excitatory amino acids (Morari et al., 1998), especially by its interactions with glutamatergic systems (Hoyt et al., 1997; Kalivas and Duffy, 1997) and directly *via* DA oxidation to a quinone moiety (Stokes et al., 1999). Like the hypoxic/ischemic mammal, the frog is also unable to prevent massive DA release into the extracellular space during anoxia. DA levels had nearly tripled within the first hour of anoxia (though the time course of this response was variable and thus not statistically significant); by 2 h, DA levels had increased to 392% of basal, and to more than 1200% of basal by 4 h of anoxia. The dramatic but rather slow increase (*vs.* depolarization) in extracellular DA levels may reflect ATP depletion; such a relationship has been demonstrated in rat nerve terminals in which the decline in ATP levels and inhibition of Na⁺/K⁺-ATPase promoted a reversal of neurotransmitter transporters (Santos et al., 1996). (Though it is interesting to note that this does not appear to occur with glutamate, whose uptake is also ATP-dependent). The DA

increase is quite distinct from what is seen in the anoxia-tolerant turtle, which prevents such a catastrophic increase by a combination of reduced efflux and continued uptake (Milton and Lutz, 1998; S. L. Milton and P. L. Lutz, manuscript in preparation). The frog model may thus be an interesting model in which to examine the mechanisms of DA failure in early anoxia, which occur so rapidly in the mammal but over a period of hours in the frog.

In summary, then, the anoxic frog is a potentially rich model of 'slow death' in which to examine the mechanisms of anoxic neuronal failure that are so similar to those which occur in the hypoxic/ischemic mammalian brain. The extended time course of ATP depletion, excitotoxin release and anoxic depolarization in the frog compared to the mammal provides a large window of opportunity within which to define and manipulate the critical stages of anoxic failure. While there are some significant differences between the mammalian and frog brain, such as the lack of increase in extracellular glutamate levels, even these differences could provide a rich area for further investigation. The frog brain, at any rate, is clearly not like those of true facultative anaerobes such as the crucian carp and freshwater turtle, which defend ATP levels and ion homeostasis and thus avoid the catastrophe of anoxic vulnerability.

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