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Neurotransmitter Levels and Energy Status in Brain of Fish Species With and Without the Survival Strategy of Metabolic Depression

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ABSTRACT. The effects of anoxia were studied in the whole brain of three fish species, each with a specific metabolic strategy for anoxic survival. Goldfish (*Carassius auratus*) combine a lactate to ethanol conversion with a metabolic depression, tilapia (*Oreochromis mossambicus*) use an anaerobic glycolysis with the strategy of metabolic depression, and carp (*Cyprinus carpio*) use an increased anaerobic glycolysis for energy production. Tilapia and carp were exposed to anoxia until they lost equilibrium and exhibited escape reactions, this occurred after 2 hours of anoxia for tilapia and 30 minutes of anoxia for carp. Goldfish were exposed to a selected period of 8 hours anoxia. The energy status and neurotransmitter (amino acid) levels in whole brain tissue were measured after anoxia exposure. The energy status was affected in all three groups exposed to anoxia. Lactic acid levels increased five- to six-fold in all three groups. No direct correlation was observed between energy status and survival strategy. Remarkably, the changes in the amino acid patterns in whole fish brains show the greatest changes in the anoxia-tolerant goldfish, an intermediate pattern in tilapia, and nearly no changes in the anoxia-intolerant carp. The changes in amino acid are probably dependent on the period of anoxia exposure. For goldfish, the lactate-ethanol conversion primarily determines anoxic survival, but the strategy of metabolic depression observed in goldfish and tilapia may contribute secondarily to anoxic tolerance. It is hypothesized that a decrease of excitatory neurotransmitters (mainly glutamate), in combination with an increase of inhibitory neurotransmitters (mainly GABA), may contribute to the process of metabolic depression and prolong survival. COMP BIOCHEM PHYSIOL 114A;2:189–196, 1996.

KEY WORDS. Fish, brain, amino acids, GABA, glutamate, energy status, anoxia, metabolic depression

INTRODUCTION

The mammalian brain is highly vulnerable to oxygen deprivation. In the absence of adequate oxygen, the increased anaerobic glycolysis (Pasteur effect) is insufficient to maintain a high-energy status. Consequently, ion pumps are affected and ionic homeostatis is lost, leading to an accumulation of extracellular K^+, which in turn causes a depolarization of the neuronal membrane. The depolarization opens the voltage-dependent Ca^{2+} channels, and an increase in cystolic Ca^{2+} occurs. Moreover glutamate accumulation occurs due to reversal of re-uptake mechanisms due to ionic gradient collapse (31). It is thought the excitatory amino acid (EEA), glutamate and aspartate, mediate tissue damage (excitotoxic damage or cell death) by opening Ca^{2+} channels. More precisely, injury of cultured cortical neurons after exposure to glutamate can be divided into two components: 1) neuronal swelling probably due to the influx of extracellular Na^+, accompanied passively by the influx of Cl^- and water; 2) delayed neuronal disintegration probably due to opening a Ca^{2+} channel by activating the *N*-methyl-D-aspartate (NMDA) receptor (7). The resulting Ca^{2+} influx results in the stimulation of phospholipid hydrolysis with a rise of harmful phospholipases leading to the formation of free radicals (16,17). These changes are irreversible if they last for more than a few minutes. Most vertebrates therefore die after a short period of anoxia. This vulnerability is characteristic for vertebrates in general and is probably the result of the brain's high energy demand. This high energy demand is apparent from the observation that brain tissue represents 0.1–1% of the body weight of vertebrates (excluding primates), but is responsible for 1.5–8.5% of the total body energy consumption in endothermic vertebrates, and a similar figure (2.7–7.4%) is found for ectothermic vertebrates (19).

Unlike mammals, some fishes can survival anoxia for a remarkably long period of time. The ability of some fish

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species to withstand severe anoxic conditions for an extended period of time can mainly be ascribed to a depression of the metabolism (39). Inhibiting neurotransmitters like GABA and glycine are active in anoxia-tolerant species (20,22,24,25).

Fish are an excellent model for studying the effects of anoxia tolerance on energy metabolism and of neurotransmitter release in brain tissue, because there is a wide scale of variation in anoxia tolerance among species. In this study, three species were used: goldfish (*Carassius auratus*), tilapia (*Oreochromis mossambicus*), and carp (*Cyprinus carpio*), each species possessing a different tolerance to anoxic survival. Goldfish are able to survive anoxia for 16 hours at 20°C (35) by metabolic depression (39) and by converting lactic acid from muscle tissue to ethanol (38), which is lost across the gills by diffusion. Tilapia shows an intermediate response and can withstand anoxia for 2 to 3 hours through a metabolic depression and increased anaerobic glycolysis, while carp shows a low anoxia tolerance of approximately 30 minutes by only increasing anaerobic glycolysis (37).

In the last 15 years, a large number of studies led to the conclusion that several of the free amino acids in the central nervous system (CNS) have neurotransmitter functions like glutamate, glutamine, glycine, taurine, alanine, γ-aminobutyric acid, aspartate and serine (18,36).

We hypothesize that anoxia-tolerant species (like goldfish) show a greater increase of inhibiting neurotransmitters like GABA and glycine and decrease of excitatory amino acids like glutamate than do less anoxia tolerant species. Furthermore, we assume that [ATP] and energy status are maintained in anoxia tolerant fish, while this is not the case in anoxia-intolerant species.

The aim of this study was to observe changes in amino acid (neurotransmitter) patterns and energy status in the brain of three different fish species, each with its own specific anoxia tolerance. Because the brain is the most vulnerable of all organs for oxygen deprivation, this study has ecological implications concerning the explanation of survival strategies in fish species exposed to conditions of low oxygen.

MATERIAL AND METHODS

Animals

Tilapia (*Oreochromis mossambicus*) were obtained from the Catholic University Nijmegen (The Netherlands), carp (*Cyprinus carpio*) from the Agricultural University Wageningen (The Netherlands), and goldfish (*Carasius auratus*) from a commercial fish dealer (Boon, Hardinxveld, The Netherlands). The bodyweight of the tilapia, carp, and goldfish group were respectively 302.1 ± 138.5, 581.9 ± 104.5 and 305.6 ± 149.4 grams. The animals were acclimated to normal oxygen levels (80–100% air saturation), a 14-hour light period, and a temperature of 20°C; they were fed daily with Trouvit pelleted feed (Trouw, Putten, The Netherlands).

Induction of Anoxia

The animals were starved for 2 days in a 35-liter flow-through respirometer at normal oxygen levels (85% air saturation) (35). The supply of water to the respirometer was regulated by an EIL oxygen controller (EIL LTD Richmond, Surrey, England, model 9402) connected to a valve. Oxygen tension was graphically registered on a recorder. The temperature in the experimental set up was kept at 20 ± 0.5°C. On the third day, anoxia was introduced by stopping the supply of freshly aerated water to the respirometer. The moment of anoxia was estimated according to the extrapolation procedure of van den Thillart *et al.* (34). It took approximately 2 hours before anoxia in the water was reached. The tilapia were exposed to anoxia for 2 hours, the carp 30 minutes, and the goldfish 8 hours. The criterion for the exposure duration for carp and tilapia was the observed change of behaviour of the animals in the respirometer (see results). For goldfish the 8-hour exposure period was defined beforehand.

Sampling Procedure

After exposure to anoxia, a solution of the anaestheticum 3-aminobenzoate ethyl ester methanosulphonate (MS-222, Sigma, St Louis, MO, U.S.A.) at a final concentration of 100 ppm was injected in the respirometer. The whole brain was removed and directly freeze-clamped between a pair of aluminum clamps cooled in liquid nitrogen. The samples were stored at −180°C until extraction.

Tissue Preparation

Frozen tissue was powdered in an automatic grinder (Retsch type RMO, Haan, Germany) with liquid nitrogen and 4.0 volumes of perchloric acid (8%, v/v) in ethanol (40%, v/v) containing 4 mM NaF and 10 mM EDTA. The powder was stored for 15 minutes at −20°C in a centrifuge tube. Thereafter, the power was further homogenized on ice with a high-speed mixer (Salm & Kip BV, type X 1020, Dottingen, Germany). The homogenate was stored for 30 minutes on ice and was further centrifuged (Sorvall RC-5B) for 20 minutes at 30,000 **g**. The extract was neutralized to pH 7.0 with 3 M potassium carbonate in 0.5 M triethanolamine. Finally, the extracts were cooled and centrifuged for 20 minutes at 30,000 **g**. The extracts were divided over Eppendorf tubes and stored at −180°C until analysis.

HPLC-Analysis of Nucleotides

Nucleotides were measured in the same brain tissue extracts that were used for the amino acid analysis. Nucleotide analysis was based on the HPLC-method of Harmsen *et al.* (10). The HPLC configuration consisted of a LKB 2248 pump with a Pharmacia LKB low-pressure solvent mixer. The separation was performed on an anion-exchange column (200 × 4.6 mm) packed with 10 μm Partisal SAX (Whatman,

Clifton, N.J., U.S.A.) operating at room temperature. A gradient elution system was used consisting of eluent A: 0.01 M H_3PO_4 adjusted to pH 2.85 and eluent B: 0.75 M KH_2PO_4 adjusted to pH 4.40. The gradient profile was 0% of B during the first 5 minutes, followed by a linear gradient from 0 to 100% B for t = 5–35 min, 100% B for t = 35–40 min, and 0% B at t = 45 min. Detection was performed using an UV dual-wavelength detector (LKB 2141 monitor) set at 256 and 210 nm. Quantification was performed using external standards.

Detection of Lactic Acid

Lactic acid was detected enzymatically according to the method of Hohorst (13).

HPLC Analysis of Amino Acids

The amounts of amino acids in the extracts were determined by reverse-phase high-performance liquid chromatography (HPLC) with UV detection at 330 nm. Amino acids were derivatized with *o*-phthaldialdehyde (OPA) as described by van der Boon *et al.* (3). The derivatization reagent for the amino acids was prepared fresh every day. 7.6 mg of *o*-phthaldialdehyde was dissolved in 300 μl of methanol. Next, 2.2 ml of the 0.4 M sodium tetraborate buffer (pH 10.2 with 4 M sodium hydroxide) and 10 μl of 2-mercaptoethanol (2-ME) were then added. The mixture was filtered through a Millex-HV4 filter (Millipore). To 180 μl of the sample 20 μl of an internal standard 12.5 mM 2-aminobutyric acid (2-ABA) was added. In this way, a correction can be made for the variation in derivatization among samples. Using an automatic mixing routine with the LKB autosampler, 10 μl of the sample-ABA mixture was mixed with 30 μl of the OPA derivatization reagent for exactly 1 minute. Thereafter, 10 μl was injected into the HPLC column. The HPLC system consisted of an LKB 2248 pump with a Pharmacia LKB low-pressure solvent mixer. Separation was performed using a reverse-phase column (4.6 mm × 7.5 cm), Ultrasphere ODS 3 μm (Beckman Instruments, Inc.), operating at room temperature. The flow rate was 1 ml.min^{-1} and the eluent consisted of 65% A (60 mM K_2HPO_4, 30 mM citric acid, 0.36 mM EDTA with the mixture set at pH 6.3) and 35% B (methanol) (20). Quantification was performed using external standards.

Statistics and Calculations

Statistics were performed using a one-way ANOVA. Normality of the data and homogeneity of variances were checked by Kolmogorov-Smirnov and F_{max} tests, respectively. $P \leq 0.05$ was considered as statistically significant. For the nucleotides, the following parameters were calculated:

* Total pool of adenine nucleotides:

$$TAN = [(ATP + ADP + AMP)],$$

* the adenylate energy charge:

$$AEC = [(ATP + \tfrac{1}{2}ADP)/(ATP + ADP + AMP)],$$

* the IMP-load, IL = [(IMP)/(ATP + ADP + AMP)].

RESULTS

Behavioural Changes During Anoxia

The following observations were made on the behaviour of the fish during nomoxia and anoxia. The animals were kept for 2 days in the flow-through respirometer to minimize stress effects. During this period, all species showed normal behaviour, that is, slow swimming activity alternated with periods of rest and quiet ventilation. Only tilapia showed social interactions with a characteristic hierarchy. During the hypoxic transit period, the ventilation frequency increased. During the anoxic period, tilapia had normal body position for all but the end of this period. Only goldfish were laying quietly at the bottom of the respirometer. At the end of the anoxic period, the carp and tilapia had lost equilibrium and showed, from time to time, escape reactions characterized by sudden jumping movements.

The difference in survival time to anoxic conditions of the three subspecies is remarkable: only 30 minutes for carp, 3 hours for tilapia and more than 8 hours for goldfish. Goldfish can resist anoxia for an even longer time. Periods of anoxic survival up to 16 hours at 20°C have been reported (33).

Energy Status and Lactic Acid

Nucleotides, lactic acid concentration, and calculated parameters TAN, AEC, and IL for the three species after normoxia and anoxia are given in Table 1. The energy status was affected in all three groups after exposure to anoxia, which can be concluded from the depletion of phosphocreatine, the drop of [ATP], changes for ADP, AMP and IMP, the decline of the adenylate energy charge (AEC) and the rise of the IMP-load (IL). Phosphocreatine levels were significantly lowered for goldfish, tilapia and carp after anoxia until 6.3%, 26.2%, 21.6% of the initial normoxic value. In the goldfish group, [ATP] declined significantly after anoxia to 56.2% of the initial value, while the minima for tilapia and carp were, respectively, 81.3% and 59.8% of the normoxic value (not significant). The corresponding adenylate energy charge (AEC) in all three groups during normoxia was 0.74–0.78. In goldfish, the AEC was significantly lowered after 8 hours of anoxia to 0.53. In tilapia and carp, the AEC was not significantly lower after anoxia, although the animals were already severely stressed by the asphyxic conditions; the value declined for tilapia and carp after anoxia to 0.75 and 0.61, respectively. IMP and IL increased in all three groups, but for carp these values were significantly higher. The IMP levels after anoxia for goldfish, tilapia and

TABLE 1. Energy status and lactic acid accumulation in brain tissue of three fish species. For each species, five individuals were measured. *Denotes a significant difference ($P \leq 0.05$)

	Goldfish-Normoxia (mM)	Goldfish-Anoxia (mM)
PCr	3.159 ± 1.25	0.199 ± 0.40*
ATP	0.441 ± 0.08	0.248 ± 0.08*
ADP	0.267 ± 0.039	0.392 ± 0.153
AMP	0.067 ± 0.037	0.188 ± 0.055*
IMP	0.151 ± 0.060	0.383 ± 0.274
NAD^+	0.179 ± 0.079	0.160 ± 0.042
TAN	0.776 ± 0.093	0.827 ± 0.204
EC	0.740 ± 0.045	0.533 ± 0.098*
IL	0.194 ± 0.067	0.514 ± 0.418
Lactic-acid	2.42 ± 1.143	12.44 ± 4.180*
	Tilapia-Normoxia (mM)	Tilapia-Anoxia (mM)
PCr	4.27 ± 1.10	1.12 ± 1.24*
ATP	0.397 ±0.078	0.308 ± 0.119
ADP	0.156 ± 0.035	0.145 ± 0.068
AMP	0.051 ± 0.030	0.052 ± 0.018
IMP	0.498 ± 0.220	1.037 ± 1.208
NAD^+	0.065 ± 0.067	0.042 ± 0.052
TAN	0.585 ± 0.075	0.505 ± 0.092
EC	0.777 ± 0.056	0.747 ± 0.076
IL	0.854 ± 0.361	1.839 ± 1.856
Lactic-acid	1.09 ± 0.239	5.62 ± 1.970*
	Carp-Normoxia (mM)	Carp-Anoxia (mM)
PCr	3.29 ± 1.22	0.71 ± 0.63*
ATP	0.629 ± 0.184	0.376 ± 0.169
ADP	0.320 ± 0.080	0.351 ± 0.184
AMP	0.103 ± 0.034	0.128 ± 0.017
IMP	0.190 ± 0.084	0.888 ± 0.502*
NAD^+	0.178 + 0.023	0.160 ± 0.036
TAN	1.052 ± 0.227	0.854 ± 0.327
EC	0.741 ± 0.052	0.611 ± 0.114
IL	0.176 ± 0.043	1.076 ± 0.427*
Lactic-acid	1.67 ± 0.910	9.69 ± 1.439*

carp reached, respectively, 253.6%, 208.2% and 467.4% of the initial normoxic value, while the corresponding IMP loads were 264.9%, 215.3% and 611.4%. Lactic acid increased for all three groups significantly after anoxia, with maxima for goldfish, tilapia, and carp after 514%, 516% and 580%, respectively.

Brain Amino Acids

The concentrations of amino acids of the three fish species during normoxia and after anoxia exposure are given in Table 2. Alanine, as an endproduct of anaerobic metabolism, accumulates during anoxia in all three species. For goldfish and tilapia, there is a significant difference between the normoxic and anoxic conditions, the increase for these species was 68.0% and 39.8%, respectively, while for the carp the alanine level increased only 10.8% (not significantly different) (Table 2).

TABLE 2. Amino acid patterns in brain tissue of three fish species. For each species, five individuals were measured. *Denotes a significant difference ($P \leq 0.05$)

	Goldfish (mM) Normoxia	Anoxia
Glutamate	3.76 ± 0.34	2.90 ± 0.20*
Glutamine	2.58 ± 0.41	1.93 ± 0.09*
Glycine	2.43 ± 0.23	3.05 ± 0.16*
Taurine	6.80 ± 0.83	7.59 ± 0.58
Alanine	1.50 ± 0.14	2.52 ± 0.11*
γ-Aminobutyric acid	2.42 ± 0.25	3.60 ± 0.12*
Aspartate	0.29 ± 0.11	0.29 ± 0.06
Serine	0.53 ± 0.09	0.69 ± 0.10*
	Tilapia (mM) Normoxia	Anoxia
Glutamate	4.04 ± 0.29	4.37 ± 0.53
Glutamine	3.46 ± 0.51	3.04 ± 0.28
Glycine	3.15 ± 0.10	3.85 ± 0.47*
Taurine	11.91 ± 1.40	12.02 ± 1.87
Alanine	1.81 ± 0.13	2.53 ± 0.35*
γ-Aminobutyric acid	3.11 ± 0.18	3.97 ± 0.45*
Aspartate	0.25 ± 0.14	0.41 ± 0.15
Serine	0.64 ± 0.04	1.03 ± 0.15*
	Carp (mM) Normoxia	Anoxia
Glutamate	3.49 ± 0.35	3.23 ± 0.30
Glutamine	4.77 ± 1.54	2.29 ± 0.20*
Glycine	2.29 ± 0.14	2.33 ± 0.22
Taurine	6.15 ± 0.35	6.08 ± 0.31
Alanine	1.30 ± 0.07	1.44 ± 0.11
γ-Aminobutyric acid	2.11 ± 0.12	2.34 ± 0.16
Aspartate	0.22 ± 0.11	0.10 ± 0.05
Serine	0.65 ± 0.09	0.74 ± 0.15

The concentration of glutatmate decreased for goldfish significantly, while for tilapia and carp there was no significant difference between normoxic and anoxia conditions (Table 2). The concentration of the other excitatory amino acid aspartate shows no significant difference for all three species (Table 2). Glutamine, the precursor of glutamate, shows a decrease in all three species. For goldfish and carp, this decrease is significantly different. The drop was 25.2%, 12.1% and 52.0% for goldfish, tilapia and carp, respectively (Table 2). GABA levels after anoxia were significantly higher for goldfish and tilapia and showed for those species a rise of 48.8% and 27.7%, respectively. For the anoxia-intolerant carp the rise of GABA was 10.9% after anoxia but was not significantly different (Table 2).

Glycine levels were significantly higher in goldfish and tilapia after anoxia and increased for those species by 25.5% and 22.2%, respectively. In carp, the glycine level was not significantly different between the two conditions (Table 2).

Taurine showed no significant changes. In goldfish and tilapia there were increases of 11.6% and 0.9%, respectively, while in carp there was a decrease of 1.1% (Table 2).

Serine shows a significant increase in goldfish and tilapia, while this is not significant for carp. For goldfish, tilapia and carp, the increase was 30.2%, 60.9% and 9.0%, respectively (Table 2).

DISCUSSION

Energy Status in Whole Brain

By comparing the energy status in whole brains with results from two earlier studies in our laboratory, the following conclusion can be made. In short, the normoxic values agree reasonably well. PCr levels were in the range of 2.5–5.8 mM, [ATP] in the range of 0.4–1.0 mM, [ADP] in the range of 0.27–0.48 mM, TAN values in the range of 0.6–1.4 and AEC values in the range of 0.75–0.83. The IMP and IL levels, however, were higher in our study than in the two earlier studies (5,27).

The goldfish and carp data can be compared with previous anoxia studies (5,27). For the goldfish in our study, the energy status was more affected by anoxia, based on the depletion of PCr, the drop of ATP and AEC and the severe rise of IMP and IL. In contrast, the animals in the study of van der Boon *et al.* (5) were able to maintain ATP levels and AEC. This can probably be explained by the longer transition phase of 3 hours, which was used in the study of van der Boon *et al.* (5) to reach anoxia. This enabled the animals to adapt to the anoxic conditions. In contrast, the transition phase took 1–2 hours in our studies perhaps leading to the drop of the AEC and [ATP]. For carp, the energy status was more affected in the study of van Raaij *et al.* (27), but the time of anoxic exposure was longer (1.5 hours of anoxia), while in our studies the animals were already severely stressed due to asphyxia after 30 minutes. Again, the transition period to anoxia was longer in the study of van Raaij *et al.* (27): 7.75 kPa per hour, which took approximately 3 hours to reach anoxia.

The lactate concentrations in brain tissue of the three species is inversely proportional to the depletion of PCr, the drop of [ATP] and the AEC. In goldfish, the PCr are most depleted and the [ATP] and ACE dropped sharply while the [lactate] reached its maximum. Carp takes for energy stores an intermediate position, while tilapia is less affected energetically. Consequently, the sequence for lactate accumulation, divided by the exposure time, for goldfish, tilapia and carp were respectively, 0.026, 0.047 and 0.323 mM per minute, in order of descending sequence: carp > tilapia > goldfish.

Surprisingly, the energy status of the whole brain in the anoxia-tolerant species goldfish and tilapia was also affected by anoxia in this study. Based on the drop of [ATP] and the decline of the AEC (Table 1), the energy status was more affected for goldfish than for tilapia. This can be explained by the fact that the goldfish were exposed to anoxia for 8 hours while tilapia was exposed for only 2 hours. Our initial hypothesis was that the energy status of anoxia-tolerant species would be unaffected. An important result of this study is that the anoxia-tolerant goldfish are able to survive anoxia, although the AEC of a vital organ like the brain is impaired. In contrast, the AEC of tilapia and carp is almost unaffected, although the animals were already severely stressed due to asphyxic conditions. It can be concluded from these results that the energy status of the brain tissue is not a valid parameter to use for determination of the anoxia tolerance of a species. Anoxia tolerance of a species may be caused by three factors: the tolerance of a species for the accumulation of metabolites, the amount of energy stores, and the efficiency of the energy consumption process.

Moreover, the fall of [ATP] and AEC in the goldfish brain may reflect a lowered turnover rate of ATP (giving a lower steady state concentration of ATP) due to metabolic depression. Thus, energetically, the animal is not compromised. By contrast, for the carp, which does not show a metabolic depression, the lower ATP level is a result of an inability to maintain ATP production. The low ATP level may become a serious problem for the animal since the ATP utilizing processes (notably the Na/K pump) are still running on normal speed and are therefore in need of a normal ATP supply to maintain ionic gradients, etc. The tilapia can probably be considered as an intermediate between these two extremes.

Amino Acids in Whole Brain

It must be noted that neurotransmitter levels in whole brain do not correspond to intrasynaptic concentrations. Excitatory, as well as inhibitory neurotransmitters are only effective when they are released from their intracellular stores. In general, extracellular levels of neurotransmitters are about 1/1000 of those found inside brain cells (23). Recently, with microdialysis, *in vivo* measurements of neurotransmitter concentrations were performed in the extracellular space of anoxic turtle brain (*Trachemys scripta*) (23,24). Again, as in this study, a large increase of the inhibitory neurotransmitter GABA in whole brain during anoxia was reported (24). Because fish brains are so small (0.3–0.5 grams) the question remains whether microdialysis is applicable for studying extracellular neurotransmitter levels in fish brains. Therefore, the measurement of neurotransmitter levels in whole brain, as performed in this study, is a first attempt to explore this area: survival strategies of different fish species in relation to brain neurotransmitter levels.

The levels of amino acid in crucian carp brains during normoxia can be estimated from Table 2 (25). GABA, glycine, alanine, taurine, glutamate, and glutamine levels during normoxia are 1.29, 0.59, 0.25, 6.71, 5.89, and 2.05 mM, respectively. After 17 days of anoxia, Nilsson *et al.* (25) detected GABA, glycine, alanine, taurine, glutamate and glutamine levels of 5.82, 1.33, 1.24, 7.49, 2.95 and 0.61 mM, respectively. These values correspond to those mea-

sured in our study of normoxic and anoxic goldfish brains (Table 2).

Remarkably, the changes in the amino acid patterns in whole fish brain show the greatest changes in the anoxia-tolerant goldfish. Significant changes were observed for glutamate, glutamine, glycine, alanine, γ-aminobutyric acid, and serine. Tilapia shows an intermediate response, while significant changes were observed for glycine, alanine, γ-aminobutyric acid and serine. Carp shows minor changes; only the glutamine level is significantly different.

The observed changes may be caused by the anoxic conditions. For example, alanine shows an accumulation under anoxia conditions because it is an endproduct of anaerobic metabolism. In many organisms and tissues, alanine accumulation has been observed: in turtle brain (25) and liver (2), in trout and carp red muscle (15), in whole goldfish (34), and in whole blood, liver, white muscle and red muscle of anoxia goldfish (4). The accumulation of alanine can be explained by the fact that the enzyme glutamate pyruvate transaminase is operative in these tissues (4).

GABA accumulation under anoxia has been observed in many organisms. In brain of the anoxia tolerant turtle (*Trachemys scripta*) (12), in anoxia-tolerant loggerhead sea turtles (*Caretta caretta*), and crucian carp (*Carassius carassius*) (25), but also in anoxia-intolerant species like brown anole lizards (*Anolis sagrei*) (25) and dogs (32). GABA accumulation is accompanied by glutamate depletion (26). Two causes can be responsible for the glutamate depletion. First, under anaerobic conditions, the conversion of glutamate to GABA catalyzed by glutamate decarboxylase will continue (26,29). Secondly, glutamate synthesis from glutamine and α-ketoglutarate will slow down or stop under anaerobic conditions (6,26). GABA accumulation is caused by two processes. First, glutamate to GABA conversion continues during anoxia (26,29). Secondly, GABA breakdown is catalyzed by the NAD-dependent enzymes GABA aminotransferase and succinic semialdehyde dehydrogenase and needs α-ketoglutarate, a product of TCA-cycle activity. In addition, NAD is in short supply during anoxia. However, it is assumed the TCA-cycle is not operative under anaerobic conditions, which results in a halt of GABA breakdown (26).

GABA is the main inhibitory neurotransmitter, and glutamate is the main excitatory neurotransmitter. Binding of GABA to GABA receptors will increase Cl^- and K^+ conductance and decrease Ca^{2+} conductance (26). Consequently, the membrane potential remains at the resting potential or can even be hyperpolarized, counteracting excitatory processes like membrane depolarization and opening of voltage dependent Ca^{2+} and Na^+ channels (26). In contrast, the excitatory neurotransmitter glutamate activates receptors that mediate opening of Ca^{2+} and Na^+ channels, resulting in a Ca^{2+} influx. This is followed by stimulation of phospholipid hydrolysis with a rise of destructive phospholipases causing brain damage (26).

The question remains how goldfish and tilapia are able to endure at least a few hours of anoxia, while carp die after only 30 minutes. For goldfish, the major survival mechanism to withstand anoxia for such an extended period is the lactate-ethanol conversion mechanism (38), but another mechanism may also be important. It has become clear from direct calorimetric studies that goldfish can reduce their metabolic rate under anoxic conditions to 30% of the normoxic metabolic rate, resulting in the so-called metabolic depression (8,39). Tilapia, on the other hand, reduce the metabolic rate during severe hypoxia to 50% of the normoxic metabolic rate (9). From a previous ^{31}P-NMR study exposing the three fish species (goldfish, tilapia and carp) to anoxic conditions, it became clear from the slow depletion of PCr stores in muscle tissue that goldfish and tilapia had reduced their metabolic rate by metabolic depression, while the PCr flux in carp muscle was much faster (37).

It is clear that inhibitory amino acids are important for brain metabolism. Because the brain is only 0.3% of the body weight in fish, how is an energy preservation of 70% of the normoxic metabolic rate possible if only the brain is affected? The answer is two-fold. Firstly, in other tissues like heart and intestine, peripheral GABAergic systems were observed; these may play a role in peripheral energy conservation (14,26). Secondly, the brain can downregulate the systemic energy consumption through the peripheral nervous system and/or hormones. In some studies, extracts have been isolated that drive animals to a hypometabolic state after injection. This was observed for insects in diapause (40), lungfish in estivation (30) and mammals in hibernation (11). However, the metabolic depression, which lasts only a few hours, is probably a different condition from diapause, estivation or hibernation, which all take place on a scale of weeks to months. Probably GABA has an inhibiting effect on the sympathetic nervous system. As a consequence, the animals are fully relaxed and show no muscle tension. This could explain the large reduction of energy consumption and might be the basis for the process of metabolic suppression.

GABA accumulation (to a lesser extent) also occurs in anoxia-intolerant species like the carp in this study, the brown anole lizard (*Anolis sagrei*) (25) and the dog (32). What indications do we have that the inhibitory neurotransmitter GABA is involved in the process of metabolic depression? First, using the ethanol excretion of crucian carp as an index for anaerobic metabolic rate, the use of a GABA-blocker isoniazid, an inhibitor of GABA synthesis, increased the anaerobic metabolic rate 3-fold. Second GABA is also active in endotherms. In rats injected with the GABA-receptor agonist muscimol, the decrease of body temperature was dose-dependent (26,28). Third, a decrease in body temperature of 3°C was observed in gerbils when GABA levels were increased pharmacologically with gammavinyl GABA, and depletion of energy stores in brain was retarded (1,28).

Other compounds or amino acids may contribute to the survival strategy of metabolic depression. Aminophylline, an adenosine antagonist, increased the rate of anaerobic ethanol excretion (an index for anaerobic metabolic rate) in crucian carp 3-fold without affecting the normoxic oxygen consumption (normoxic metabolic rate) (21). In other words, an adenosine blocker increased the anaerobic metabolic rate 3-fold, probably via inhibition of the metabolic depression process. Other inhibitory amino acids (taurine and glycine, for example) also may play a role. In this study, only significant increases were observed for glycine in goldfish and tilapia.

It is strange that anoxia-intolerant species like brown anole lizards (*Anolis sagrei*) (25) and dogs (32) die so quickly, while the mechanisms of GABA accumulation and glutamate depletion (to a lesser extent) also occur in these species. The animals are presumably unable to prevent a drop in energy status of the brain by increased anaerobic glycolysis, so they die before they can profit from the protective mechanisms caused by the changes in amino acids in brain, GABA accumulation and glutamate decrease.

For the fish species crucian carp, Nillson (20,22) has demonstrated an accumulation of the inhibiting neurotransmitter GABA and a decrease of the excitatory neurotransmitter glutamate during anoxia. In this study, these results are confirmed for another anoxia-tolerant species, the goldfish, while the data are coupled to the energy status of brain tissue. Interestingly, those changes in amino acids are not observed in the anoxia-intolerant carp, while tilapia shows an intermediate response.

In conclusion, to understand the changes in brain during anoxia, we studied the changes in amino acids and energy status of anoxia-tolerant and anoxia-intolerant fish species. The energy status of the brain was affected in all three species, anoxia-tolerant as well as anoxia-intolerant. Significant changes in amino acid patterns were observed in anoxia-tolerant species. It is assumed that these changes in amino acid patterns are caused by the anoxic conditions, but, on the other hand, these changes also may contribute to an increased anoxia tolerance.

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References

1. Abel, M.S.; McCandless, D.W. Elevated γaminobutyric acid levels attenuate the metabolic response to bilateral ischemia. J. Neurochem. 58:740–744;1992.
2. Arillo, A.; Balletto, E.; Cherchi, M.A. Environmental adaptation: free amino acids in the liver of anoxic vertebrates. Boll. Mus. Ist. Univ. (Genova) 43:115–121;1975.
3. Boon van der, J.; Van den Thillart, G.E.E.J.M.; Addink, A.D.F. Reversed-phase liquid chromatographic analysis of o-phtaldialdehyde-derivatized free amino acids in two types of goldfish muscles. J. Pharm. Biomed. Anal. 7:471–481;1989.
4. Boon van der, J.; Eelkema, F.A.; van den Thillart, G.E.E.J.M.; Addink, A.D.F. Influence of anoxia on free amino acid levels in blood, liver and skeletal muscles of the goldfish, (*Carassius auratus* L.). Comp. Biochem. Physiol. 101B:193–198;1990.
5. Boon van der, J.; de Jong, R.L.; Thillart van den, G.E.E.J.M.; Addink, A.D.F. Reversed-phase ion-paired HPLC of purine nucleotides from skeletal muscle, heart and brain of the goldfish, *Carassius auratus* L. 11. Influence of environmental anoxia on metabolite levels. Comp. Biochem. Physiol. 101B: 583–586;1992.
6. Bradford, H.F. Chemical Neurobiology. New York: W.H. Freeman; 1996.
7. Choi, D.W. Excitotoxic cell death. J. Neurobiol. 23:1261–1276;1992.
8. Ginneken van, V.J.T.; Gluvers, A.; van der Linden, R.W.; Addink, A.D.F.; van den Thillart, G.E.E.J.M. Direct calorimetry of aquatic animals: automated and computerized data-acquisition system for simultaneously direct and indirect calorimetry. Thermochimica Acta 247:209–224;1994.
9. Ginneken van, V.; Addink, A.; van den Thillart, G.; Körner, F.; Noldus, L.; Buma, M. Locomotor activity measurements in combination with calorimetry for Tilapia exposed to normoxic and hypoxic conditions: evidence that metabolic depression is not caused by a reduction of locomotor activity. J. Exp. Biol. 1995: submitted.
10. Harmsen, E.; De Tombe, P.Ph.; de Jong, J.W. Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by high-performance liquid chromatography. J. Chromatogr. Biomed. Appl. 230:131–136;1982.
11. Heller, H.C.; Musacchia, X.J.; Wang, L.C.H. (eds). Living in the Cold. Amsterdam: Elsevier/Nort Holland Biomedical Press; 1986.
12. Hitzig, B.M.; Kneussl, M.P.; Shih, V.; Brandstetter, R.D.; Kazemi, H. Brain amino acid concentrations during diving and acid-base stress in turtles. J. Appl. Physiol. 58:1751–1754; 1985.
13. Hohorst, H.J. Methoden der enzymatischen analyse. Auflage Band 11 p. 1425, Verlag Chemie Weinheim/Bergstr.
14. Jessen, K.R.; Mirsky, R.; Hills, M. GABA as an autonomic neurotransmitter: studies on intrinsic GABA-ergic neurons in the myenteric plexus of the gut. Trends Neurosci. 10:255–262;1987.
15. Johnston, I.A. Anaerobic metabolism in the carp (*Carassius carassius* L.) Comp. Biochem. Physiol. 51B:235–241;1975.
16. Lassanova, M.; Tursky, T. The influence of hypoxia on the formation of amino acids from oxidized substrates in the rat brain *in vivo*. Physiol. Res. 40:403–411;1991.
17. Lutz, P.L. Mechanisms for anoxia survival in the vertebrate brain. Ann. Rev. Physiol. 54:601–618;1991.
18. McGeer, P.L.; McGeer, E.G. Amino acid neurotransmitters. In: Siegel, C.J. *et al.* (eds). Basic Neurochemistry: Molecular, Cellular, and Medical Aspects, 4th ed. New York: Raven Press, Ltd.; 1989: chap. 15.
19. Mink, J.W.; Blumenschine, R.J.; Adams, D.B. Ratio of central nervous system to the body metabolism in vertebrates: its constancy and functional basis. Am. J. Physiol. 241:R203–R212; 1981.
20. Nilsson, G.E. Long-term anoxia in crucian carp: changes in the levels of amino acid and monoamine neurotransmitters in the brain, catecholamines in chromaffin tissue, and liver glycogen. J. Exp. Biol. 150:295–320;1990.
21. Nilsson, G.E. The adenosine receptor blocker aminophylline increases anoxic ethanol excretion in crucian carp. Am. J. Physiol. 261:R1057–R1060;1991.

22. Nilsson, G.E. Evidence for a role of GABA in metabolic depression during anoxia in crucian carp (*Carassius carassius* L.). J. Exp. Biol. 164:243–259;1992.
23. Nilsson, G.E. Neurotransmitters and anoxia resistance: comparative physiological and evolutionary perspectives. In: Hochachka, P.W., *et al.* (eds). Surviving Hypoxia, Mechanisms of Control and Adaptation. Boca Raton, Fla.: CRC Press, Inc.; 1993: chap. 27.
24. Nilsson, G.E.; Lutz, P.L. Release in inhibitory neurotransmitters in response to anoxia in turtle brain. Am. J. Physiol. 261: R32–R37;1991.
25. Nilsson, G.E.; Lutz, P.L.; Jackson, T.L. Neurotransmitter and anoxic survival of the brain: A comparison of anoxia-tolerant and anoxia-intolerant vertebrates. Physiol. Zool. 64:638–652; 1991.
26. Nilsson, G.E.; Lutz, P.L. Mini review. Role of GABA in hypoxia tolerance, metabolic depression and hibernation-possible links to neurotransmitter evolution. Comp. Biochem. Physiol. 105C:329–336;1993.
27. Raaij, van, M.T.M.; Bakker, E.; Nieveen, M.C.; Zirkzee, H.; van den Thillart, G.E.E.J.M. Energy status and free fatty acid patterns in tissues of common carp (*Cyprinus carpio*, L.) and rainbow trout (*Oncorhynchus mykiss*, L.) during severe oxygen restriction. Comp. Biochem. Physiol. 109A:755–767;1994.
28. Sancibrian, M.; Serrano, J.S.; Minano, F.J. Opioid and prostaglandin mechanisms involved in the effects of GABAergic drugs on body temperature. Gen. Pharmacol. 22:259–262;1991.
29. Siesjö, B.K. Brain Energy Metabolism. Chichester: Wiley; 1978.
30. Swan, H.; Reinhard, F.G.; Caprio, D.L.; Schatte, C.L. Hypometabolic brain peptide from vertebrates capable of torpor. Cryobiology 18:598–602;1981.
31. Szatkowski, M.; Attwell, D. Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. Trends in Neuroscience 17: 359–365;1994.
32. Tews, J.K., Carter, S.H.; Roa, P.D.; Stone, W.E. Free amino acids and related compounds in dog brain: post-mortem and anoxic changes, effects of amoninium chloride infusion, and levels during seizures induced by picrotoxin and pentylenetetrazol. J. Neurochem. 10:641–653;1963.
33. Thillart van den G.; Waarde van, A. Teleosts in hypoxia: aspects of anaerobic metabolism. Molec. Physiol. 8:393–409; 1985.
34. Thillart van den, G.; Kesbeke, F.; Waarde van, A. Influence of anoxia on the energy metabolism of goldfish (*Carassius auratus* L.). Comp. Biochem. Physiol. 55A:329–336;1976.
35. Thillart van den, G.; Verbeek, R. Anoxia induced oxygen debt of goldfish (*Carassius auratus* L.). Physiolog. Zool. 64: 525–540;1991.
36. Usherwood, P.N.R. Amino acids as neurotransmitters. In: Lowenstein, O. (ed). Advances in Comparative Physiology and Biochemistry, Vol. 7. New York: 1978:227–309.
37. Waarde van, A.; Thillart van den, G.; Erkelens, C.; Addink, A.; Lugtenburg, J. Functional coupling of glycolysis and phosphocreatine utilization in anoxic fish muscle. J. Biol. Chem. 265:914–923;1990.
38. Waarde van, A.; Thillart van den, G.; Verhagen, M. Ethanol formation and pH-regulation in fish. In: Hochachka, P.W., *et al.* (eds). Surviving Hypoxia, Mechanisms of Control and Adaptation. Boca Raton, Fla: CRC Press, Inc.; 1993: chap. 11.
39. Waversveld van, J.; Addink, A.D.F.; Thillart van den, G. Simultaneous direct and indirect calorimetry on normoxic and anoxic goldfish. J. Exp. Biol. 142:325–335;1989.
40. Yamashita, O.; Hasegawa, K. Embryonic diapause. In: Kerkut, G.A., Gilbert, L.I. (eds). New York: Pergamon Press; 1984: 408–434.