# Anoxia / reoxygenation induced cell death in crucian carp brain

Master thesis by Lisa Yuen


**Supervisors:**

Göran E. Nilsson

Christina Sørensen

Programme for Physiology

**Department of Molecular Biosciences**

Faculty of mathematics and natural sciences

**UNIVERSITY OF OSLO 2010**

## Acknowledgements

First of all I want to thank my supervisor Professor Göran Nilsson for his great advice, and for giving the opportunity to do this master thesis. I would also like to give my deepest gratitude to my fantastic co-supervisor Christina Sørensen. Without her support, help and guidance this thesis would not have been completed.

I would also like to thank all of my fellow group members for their support and help throughout this period. Xie xie Kristian Gundersen for the use of your microscope, and Siobhan for teaching me how to use it. I also wish to thank the whole physiology department which has provided a fun and interesting learning environment.

A special thanks to my wonderful friends Håvard, Medya, Ieva and Anette for putting up with me and supporting me through five years. And to my co-master student at Göran's group, hardcore Linda Bohlin, whose encouragement, companionship during long lab hours and help has been invaluable to me through this process.

I would also like to thank my mother, father, brother and in-laws for their support, and all the food they supplied me with so that neither I nor my boyfriend would starve these last two years.

And last but not least I want to express my love and gratitude to my beloved boyfriend Thomas. You have been so understanding, encouraging and patient with me through this whole process. Thank you for enduring my nagging and keeping my spirits up when I needed it the most. With you by my side I can do anything.

August 2010

Lisa Yuen

## Abstract

The crucian carp (*Carassius carassius*) has an exceptional ability to tolerate anoxia, being able to survive without oxygen for several months at low temperatures. In response to low oxygen levels the crucian carp reduces its metabolic rate, and up-regulates glycolysis to produce enough ATP to fuel cellular ATP demand. These adaptations solve the main problem encountered during anoxia, which is to supply cells with enough ATP. The brain has a very high rate of ATP use, and it is therefore especially vulnerable during anoxic conditions. In the brain of mammals, even brief periods of oxygen deprivation can induce apoptotic cell death. It is still not known if the crucian carp brain suffers brain damage after anoxic exposure. The aim of this study was therefore to examine if anoxia, and/or subsequent reoxygenation, affect the incidence of apoptotic cell death in the brain of crucian carp.

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method was applied to stain for and quantify apoptotic cells in the crucian carp telencephalon. The amount of apoptotic cells did not increase significantly after 7 days of anoxia (at 9 °C). However, when the anoxic fish were given 1 day of reoxygenation at normal oxygen levels, a 170 % increase in the number of apoptotic cells was detected. The elevated apoptosis after reoxygenation resembles the effect of reperfusion after cerebral ischemia in mammals, where reperfusion accelerates the rate of cell death.

One possibility is that anoxia initiates apoptotic pathways in the brain without leading to actual cell death until oxygen is restored. Another possibility is that anoxia in itself does not induce apoptosis, but that the following reoxygenation causes increased apoptosis. Regardless, anoxia followed by reoxygenation does cause some damage in the form of increased levels of apoptosis in the crucian carp telencephalon. This points at an hitherto unrecognized aspect of anoxia tolerance in crucian carp: the need to possess effective mechanisms to repair a damaged brain after anoxia / reoxygenation events.

# Table of Contents

## Abbreviations

| | |
|---|---|
| AMPA | - α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate |
| ATP | - adenosine triphosphate |
| DAB | - 3, 3'-diaminobenzidine |
| DNase I | - deoxyribonuclease I |
| DND | - delayed neuronal cell death |
| dUTP | - deoxy-uridinetriphosphate |
| GABA | - γ-Aminobutyric acid |
| HRP | - horseradish peroxidase |
| HSP | - heat shock protein |
| HSP 70 | - heat shock protein 70 |
| NMDA | - N-methyl-D-aspartic acid |
| PCD | - programmed cell death |
| PB | - phosphate buffer |
| PBS | - phosphate buffer saline |
| ROS | - reactive oxygen species |
| TdT | - terminal deoxynucleotidyl transferase |
| TUNEL | - TdT mediated dUTP nick-end labeling |

## Introduction

All vertebrates need oxygen to produce ATP in order to survive. In aquatic environments oxygen is not always easily available due to its low solubility and low diffusion rate in water. Thus, obtaining enough oxygen to uphold sufficient ATP levels is a common challenge for aquatic vertebrates. At normal oxygen levels (normoxic conditions) aerobic metabolism yields 36 molecules of ATP per molecule of glucose consumed. With complete lack of oxygen (anoxic conditions), the citric acid cycle and oxidative phosphorylation stop, leaving glycolysis as the only route for ATP production, resulting in only 2 molecules of ATP per molecule of glucose. Thus, the brain of most anoxic vertebrates will suffer energy failure, which leads to loss of ion gradients and the ability to maintain membrane potentials (Johansson et al., 1995). This causes a series of catastrophic events, leading to cell death (necrosis) or cell suicide (programmed cell death, apoptosis) (Lutz and Nilsson, 2004).

A few vertebrates have the ability to survive long periods of anoxia. These include the painted turtle (*Chrysemys picta*) (Ultsch and Jackson, 1982), the goldfish (*Carassius auratus*) (Shoubridge and Hochachka, 1980), and the crucian carp (*Carassius carassius*) (Johnston and Bernard, 1983, Holopainen et al., 1986, Blazka, 1958). This unique ability is required by the crucian carp to survive for prolonged anoxic periods in small ponds and lakes in the northern hemisphere. These lakes freeze over during the winter months and oxygen-transfer from the atmosphere is limited. This causes the rate of oxygen consumption by organisms living in the ponds to exceed the strongly suppressed rate of oxygen production from photosynthesis, and the environment often becomes completely anoxic during several months in the winter (Nilsson and Renshaw, 2004).

In contrast to most vertebrates, the crucian carp maintains brain ATP levels in the absence of oxygen. Firstly, it can sustain relatively high rates of glycolytic ATP production during anoxia because it builds up exceptionally large glycogen stores during the summer (Hyvarinen et al., 1985). When winter approaches, the liver glycogen store may amount to as much as 30 % of the liver mass, which in turn can make up 15 % of the body mass, making it the largest glycogen store of any vertebrate studied (Nilsson, 1990). Also, both the heart (Vornanen, 1994) and the brain (Vornanen et al., 2009) have considerable glycogen stores. It has been suggested that the glycogen reserve is the only factor that limits anoxic survival in crucian carp (Nilsson and Renshaw, 2004, Nilsson, 1990).

To avoid acidosis and lactate self-poisoning, the crucian carp produces ethanol as its major anaerobic end-product (Johnston and Bernard, 1983). It does this by combined actions of the pyruvate dehydrogenase and alcohol dehydrogenase. The ethanol production seems to be exclusively taking place in red and white skeletal muscle (Nilsson, 1988), after which it is readily transported with the blood to the gills, through which it easily diffuses into the ambient water (Nilsson, 1988, Vornanen et al., 2009, Nilsson, 2001). To allow maintained neural activity during anoxia, the blood flow to the brain doubles within the first few minutes of anoxia and is then sustained throughout the anoxic period (Lutz and Nilsson, 2004). This elevation indicates an increased rate of glucose delivery and lactate removal during anoxia.

The crucian carp cannot survive anoxia by utilizing the glycolytic strategy alone; it also employs a second strategy, metabolic depression, which involves reducing the rate of ATP consumption. As the crucian carp remains active during anoxia the degree of metabolic depression that can be attained is limited. Microcalorimetry on anoxic crucian carp brain slices (telencephalon), show a 30-40 % reduction in the metabolic rate (Johansson et al., 1995). Also a study carried out on goldfish, a close relative to the crucian carp, showed a 70 % depression of the whole body metabolic rate (measured in heat production) (Van Waversveld et al., 1989). Studies on crucian carp and goldfish have shown that some brain functions are drastically reduced. The auditory nerve is strongly suppressed (Suzue et al., 1987), and light induced responses in the optic tectum as well as in the retina are suppressed during anoxia (Johansson et al., 1997). Moreover, spontaneous physical activity is reduced by about 50 % during anoxia (Nilsson and Lutz, 2004). One mechanism utilized by the crucian carp to suppress brain electrical activity is to increase the release of the inhibitory neurotransmitter GABA (Nilsson, 1990). Reducing brain functions during anoxia probably pose no threat to the crucian carp, because no predatory fish can survive the anoxic conditions in its habitat.

It is well established that the brain is one of the most sensitive organs to energy restriction as the brain has the largest consumption of oxygen and glucose, and depends almost entirely on oxidative phosphorylation for production of ATP (Taoufik and Probert, 2008). In mammalian species, anoxic conditions in the brain can be caused by cerebral ischemia (Zemke et al., 2004). During ischemia, the blood flow to the brain is reduced or completely blocked, which causes oxygen and glucose deficiency, resulting in energy failure (Zemke et al., 2004). Within the first few minutes of anoxia, the ATP depletion causes the $Na^+/K^+$-ATPase activity to slow down and eventually stop, leading to a net outflux of $K^+$ into

the extracellular space. This rapidly leads to depolarization of the membrane, upon which $Na^+$ and $Ca^{2+}$ enter the neuron and cause massive release of excitatory neurotransmitters like glutamate (Lipton, 1999, Lutz and Nilsson, 2004). The anoxic death process is accelerated by initiation of a positive feedback loop, where released glutamate activates AMPA, NMDA and kainate receptors, which are glutamate activated cation channels causing a massive inflow of $Ca^{2+}$ into the neuron. Intracellular $Ca^{2+}$ initiates mechanisms resulting in anoxic/ischemic brain damage (Bickler and Buck, 1998, Zhang et al., 2007, Taoufik and Probert, 2008) including increased free radical formation, lipid peroxidation, membrane damage (Traystman et al., 1991, Hemmen and Zivin, 2007, Taoufik and Probert, 2008), and triggering of apoptotic pathways (Broughton et al., 2009). In addition, overactivation of ionotropic glutamate receptors promotes an increase in intracellular $Na^{2+}$ and $Cl^-$, along with passive influx of water. This water influx leads to cell swelling and formation of edema, which in turn increases the intracranial pressure, causing vascular compression and herniation, and ultimately death (Hemmen and Zivin, 2007, Nakka et al., 2008).

Originally it was thought that ischemic cell death in the brain mainly involved necrosis, an uncontrolled form of cell death. Necrosis may be induced by serious physical and chemical insults, or by extreme physiological conditions like anoxia. The series of events that are involved in necrosis include compromised membrane integrity due to ATP depletion, disruption of homeostasis, swelling and eventually cell lysis, causing leakage of cell content into the extracellular space (Chowdhury et al., 2006). Necrosis often results in an inflammatory response (Lawen, 2003, Erickson, 1997). By contrast, apoptosis or programmed cell death (PCD) is morphologically characterized by chromatin condensation, cytoplasmic shrinkage and finally nuclear and cytoplasmic fragmentation into apoptotic bodies that are removed by macrophages or other phagocytic cells (Elmore, 2007, Negoescu et al., 1996, Zhang et al., 2004). A number of studies have shown that both the intrinsic and the extrinsic apoptotic pathways operate after cerebral ischemia (Broughton et al., 2009, Rosenbaum et al., 2000, Martin-Villalba et al., 1999). Today it is generally accepted that lesions after cerebral ischemia can occur by both necrosis and apoptosis. Cell death may occur shortly after the initial insult, or after hours to several weeks later, a phenomenon referred to as delayed neuronal death (DND) (Chu et al., 2002, Holopainen, 2005). Both the duration and the severity of ischemia affects the lag time between insult and cell death (Rosenblum, 1997). It has been suggested that DND following ischemia involves cell death by apoptosis rather than necrosis (Love et al., 2000, Nitatori et al., 1995, Du et al., 1996). Also, an increase in neurons

undergoing DND occurs when the blood flow to the brain is restored after an ischemic insult (reperfusion), in anoxia-intolerant vertebrates (Li et al., 2007). A prominent example is the cells in the CA1 region in mammalian hippocampus. These are particularly sensitive to ischemia, and DND occurs days after the initial ischemic insult (Wang et al., 2004, Danielisova et al., 2009, Deshpande et al., 1992, Nikonenko et al., 2009).

It has long been assumed that anoxia tolerant vertebrates like the crucian carp effectively counteracts any deleterious effects of anoxia and do not suffer cell death in the brain. Still, this is only an assumption that has not been experimentally assessed. As anoxic/ischemic conditions and reoxygenation/reperfusion causes brain damage through apoptosis in other vertebrates, it would therefore be interesting to investigate how apoptosis is affected by both of these conditions in the crucian carp. Apoptosis is the primary mechanism for eliminating injured cells in the brain of other teleosts (Zupanc and Zupanc, 2006, Zupanc, 2009), and studies carried out on apoptosis in fish have indicated that the apoptotic pathways are functionally conserved in vertebrates (dos Santos et al., 2008, Krumschnabel and Podrabsky, 2009). Measuring the incidence of apoptosis in crucian carp under different oxygen regimes could give us insight into how an energy demanding process like apoptosis is regulated during an energy deficient period. It could also tell us whether there is any brain damage during and after anoxia in crucian carp, despite the crucian carp's extreme ability to survive anoxic conditions. Consequently the aim of this study was to examine the effects of anoxia on apoptotic cell death in the brain of crucian carp exposed to anoxia or anoxia followed by reoxygenation, by quantifying apoptotic nuclei using the TUNEL method.

## Materials and methods

*Experimental animals*

The experimental animals used were crucian carp *(Carassius carassius)* obtained from Tjernsrud pond in Oslo, Norway, with weights ranging between from 26.5 to 61.0 g (42.0 ± 2.3 g, mean ± SEM). The animals were transported to the research facilities at the University of Oslo, and kept in a 750 liter holding tank. The holding tank and experimental tanks were supplied with aerated dechlorinated Oslo tap water at 9.3 ± 0.6 °C (mean ± SD), and subjected to a 12 h light/12 h dark cycle. The fish were fed daily with commercial carp food.

*Experimental set-up and design*

The experiments were conducted using two 15 liter cylindrical PVC containers with tight fitting lids. The containers were immersed in a 750 liter holding tank filled with tap water (Figure 1). All the experimental fish were acclimatized in the containers for 2 days (20 fish in each container), during which the water in both containers was bubbled with air, and the water was changed once daily to remove waste. At the start of the anoxia exposure, the lid of one of the containers was sealed tightly, and the gas supply changed to $N_2$ to remove oxygen from the water, thus making the experimental environment anoxic. This set-up has been shown to be completely anoxic ($O_2$ level < 0.1 mg $O_2$/l) in previous experiments (Nilsson, 1989). The normoxic control container was continuously bubbled with air. The fish were not fed during acclimatization or the experimental period.

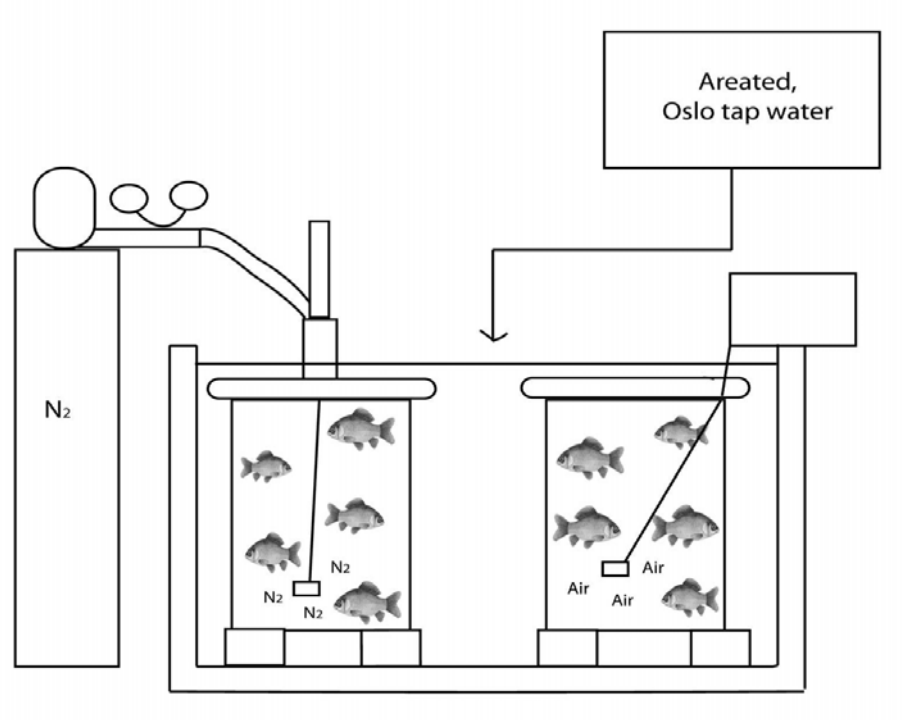


**Figure 1.** An overview of the experimental set-up. Both the anoxic and normoxic groups were kept in PVC containers submerged in a holding tank, supplied with $N_2$ and air respectively.

*Sampling and tissue preparation*

Three experimental groups were formed; normoxia 7 days (n=10), anoxia 7 days (n=10) and anoxia 7 days followed by 1 day of reoxygenation (n=10). After 7 days, 10 fish from each container were sampled (forming the normoxic and anoxic groups), and the remaining 10 fish from the anoxic container were transferred to the now empty normoxic container, where they were kept for an additional day in aerated water before sampling (forming the reoxygenation group).

The fish were killed by decapitation, whereupon the brains were dissected out and fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer (PB). After 24 h the brains were transferred to 20 % sucrose solution for another 24 h, and finally in 30 % sucrose solution for 24 h. The brains were embedded in Tissue-Tek O.C.T- medium, and frozen in isopentane cooled to its freezing point (-160 °C) in liquid $N_2$. The frozen brains were stored at -80 °C, until being sectioned at 25 µm thickness using a cryostat (Microm HM 560) and mounted on SuperFrostPlus (Thermo Scientific) slides. Slides were air dried at room temperature for 48 h and kept at -80 °C for storage.

*Apoptosis detection using the TUNEL method*

The TUNEL method is based on visualization of endonuclease activity on tissue sections, using the specific binding of terminal deoxynucleotidyl transferase (TdT) to free 3'-OH ends in the DNA. This method has been extensively used to identify apoptotic cells (Gavrieli et al., 1992, Surh and Sprent, 1994, Huppertz et al., 1999).

Unless otherwise stated, all procedures were performed at room temperature. The sections were thawed and rehydrated 5 x 5 min in phosphate buffered saline (PBS; 100 mM $NaPO_4$ set to pH 7.4). They were post-fixed in 4 % paraformaldehyde for 15 min, and then washed 3 x 5 min in PBS. Epitope retrival was done by incubation for 30 min at 85 °C in sodium citrate (0.1 M, pH 6.0) containing 0.1 % Triton X-100 (Sigma). After incubation the sections were rinsed 3 x 5 min with PBS whereupon endogenous peroxidase activity was blocked with 3 % $H_2O_2$ (Sigma) for 10 min. Sections were washed 3 x 5 min in PBS before being incubated in TdT reaction buffer (25 mM Tris-HCl, 200 mM sodium cacodylate, 0.25 mg/ml bovine serum albumin, 1 mM cobalt chloride) for 10 min, followed by incubation with the TdT reaction mixture (1600 U/ml TdT and 3.6 µM biotin-16-dUTP, both Roche Diagnostic, in TdT reaction buffer) in a humidified chamber for 1 h at 37 °C. The enzyme reaction was stopped by washing for 10 min in a stop buffer (300 mM NaCl, 30 mM sodium citrate) followed by 3 x 5 min in PBS. Slides were incubated in secondary antibody (streptavidin-HRP, BD Pharmingen™) for 20 min, and rinsed 3 x 5 min with PBS. The peroxidase activity was visualized using DAB (3, 3'-diaminobenzidine 0.01 M, Applichem, 0.01 % $H_2O_2$ in PBS, 7 min), before washing 3 x 5 min with $dH_2O$. The slides were air dried and coverslipped using Clarion ™ Mounting Medium (Sigma). Positive controls were made by incubating sections with DNase I (20 U/ml, Invitrogen) for 10 min prior to the labeling procedure to induce DNA strand breaks. For negative controls, TdT was omitted from the reaction mixture.

*Quantification of TUNEL positive nuclei*

For reasons of time limitation, only 6 out of 10 fish were examined from each group. Since the TUNEL method has been suggested to be insufficient for a definitive determination of cell death type, additional morphological analysis with light microscopy was employed. For quantification of TUNEL positive nuclei, an Olympus BX50WI microscope with a ColorView camera (1288 x 966 pixels resolution), and Olympus Cell **B** software were used. Pictures were taken at 20 x magnification and merged together using Olympus Cell$^B$ and

Photoshop CS3 software. Every fourth section (one 25 µm section per 100 µm) throughout the telencephalon was analyzed, and preoptic areas were excluded when they appeared in the same sections. Only nuclei with intact nuclear membrane, which is a characteristic morphology for apoptosis (Lawen, 2003), as well as positive TUNEL staining were counted. If only one of the two characteristics were present, the nuclei were not considered apoptotic. The staining intensity of the nuclei was also compared to the positive control, and nuclei with a lower intensity were excluded. The analyzed volume was found from the area of each section, as determined by using Photoshop CS3 software, and the section thickness (25 µm). The number of apoptotic cells per analyzed tissue volume was calculated by dividing total number of stained nuclei for all sections in a telencephalon with the total analyzed volume.

### *Statistics*

Statistical analysis was performed using STATISTICA for Windows (StatSoft, Inc., Tulsa, Oklahoma). For data on analyzed volume, a one-way ANOVA was performed. Data on weight and apoptotic nuclei per analyzed volume did not show variance homogeneity (Levene's test) and were analyzed using Kruskal-Wallis ANOVA in combination with multiple comparisons of mean ranks for all groups. Significance levels were set at $p \leq 0.05$.

## Results

### *Weight*

There was no significant difference in body weight between the groups at the time of sampling (anoxic 42.8 ± 6.2 g, normoxic 39.7 ± 3.1 g, and reoxygenation 43.3 ± 2.2 g; mean ± SD; Kruskal-Wallis ANOVA, p=0.75).

### *Cell death*

TUNEL staining was performed on brains from 18 individuals (n=6 in each group), and the staining resulted in distinctly labeled nuclei (Figure 2). Pretreatment with DNase I for formation of positive controls caused an intense staining of all nuclei in the section, and omitting TdT in the staining procedure as a negative control completely abolished all staining. There was no difference between the groups in analyzed volume (ANOVA; p=0.14). However, Kruskal-Wallis ANOVA revealed that there was a significant between-group difference in the number of stained cells per analyzed volume (p=0.002). Multiple

comparisons of ranks showed that there was no difference between the normoxic and anoxic fish (p=0.84), but the reoxygenation group had significantly more stained nuclei per analyzed volume than both the anoxic (p=0.05) and the normoxic groups (p=0.002) (Figure 3). Indeed, the number of TUNEL stained cells were 170 % higher in the reoxygenation group compared to the normoxic control.

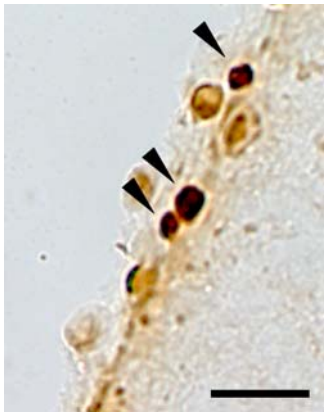

**Figure 2.** TUNEL positive nuclei in crucian carp telencephalon. The black arrows indicate the nuclei with both positive TUNEL staining and intact nuclear membrane. Scale bar 20µm.

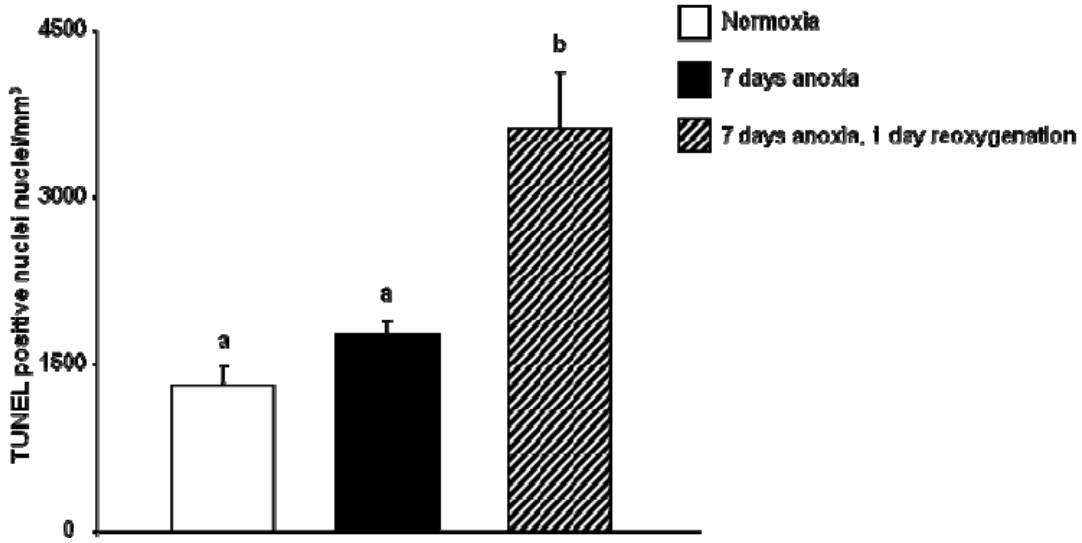


**Figure 3.** Density of TUNEL positive nuclei in the telencephalon of crucian carp exposed to 7 days of normoxia, 7 days of anoxia, and 7 days of anoxia followed by 1 day of reoxygenation. Different letters indicate a statistically significant difference between the groups. See text for ANOVA statistics. Values are means ± SEM.

## Discussion

The present study show that the incidence of apoptosis in the crucian carp telencephalon is unchanged by 7 days of anoxia (compared to normoxic animals), but a subsequent day of reoxygenation after 7 days of anoxia leads to a significant increase in cell death.

For detecting apoptotic cell death, TUNEL staining was used. This method specifically labels DNA breaks usually associated with apoptosis (Gavrieli et al., 1992). DNA fragmentation is considered to be one of the hallmarks of apoptosis (Hacker, 2000, Huang and Lu, 2001, Ucker, 1991), and is probably the most used marker for apoptosis. However, DNA fragmentation is not exclusively present in apoptotic nuclei. It is also found in late necrotic stages and the TUNEL reaction might give a positive reaction for both apoptosis and necrosis after excitotoxicity and ischemic injury (CharriautMarlangue and BenAri, 1995, Graslkraupp et al., 1995, Wolvekamp et al., 1998). We therefore assessed the staining intensity and morphological appearance of the TUNEL stained nuclei to exclude any false positives due to necrosis. Thus, believe that although there may be a few false positives stemming from necrosis (CharriautMarlangue and BenAri, 1995), the quantified cell death in this study mainly reflects apoptosis.

Characteristic apoptotic processes, like DNA alterations and cytoskeletal proteolysis require energy (Kerr et al., 1972). It has been shown that the availability of ATP and mitochondrial integrity determine whether cells die by necrosis or apoptosis (Roy and Sapolsky, 1999, Brunelle and Chandel, 2002, McClintock et al., 2002). In anoxia-intolerant vertebrates, apoptotic cell death can take place as long as ATP remains available, but when cellular energy stores are empty, cell death occurs by necrosis (Pagnussat et al., 2007). In contrast, the crucian carp is not subject to this severe ATP depletion, and as a consequence, it should be able to remove cells through apoptosis. Indeed, this was apparent in the present study, where the incidence of apoptosis was maintained after 7 days in anoxia. Zupanc (1998) showed that the majority of injured cells after a lesion in the cerebellum of the brown ghost knifefish (*Apteronotus leptorhynchus*) were removed by apoptosis, and only a few cells died by necrosis. Apoptosis is also the main mechanism for cell death during proliferative activity in both the retina and cerebellum of adult fish (Soutschek and Zupanc, 1996, Mizuno and Ohtsuka, 2009).

The telencephalon was chosen for analysis in this study as the dorsomedial area of the teleost telencephalon is believed to be homologous to the mammalian hippocampus (Saito and Watanabe, 2006, Broglio et al., 2005, Broglio et al., 2010, Vargas et al., 2009). Hippocampal functions include accommodation of memories and spatial learning (Spencer and Bland, 2007, Hodges et al., 1996). In mammals, the hippocampus has a particularly high rate of energy utilization, and thus also a high oxygen demand, explaining why it is often more severely damaged than other brain regions during anoxia (Gadian et al., 2000, Leblond and Krnjevic, 1989). Specifically the CA1 region contains cells that are particularly sensitive to ischemia, and it has been suggested that these cells die more easily by apoptosis after being subjected to ischemia than other brain cells (Kirino, 1982, Nitatori et al., 1995, Deshpande et al., 1992, Wang et al., 2004).

Lack of oxygen inhibits the electron-transport chain and causes loss of inner mitochondrial membrane potential, resulting in activation of proapoptotic factors (Brunelle and Chandel, 2002). If the cell is able to resume energy production when oxygen levels are restored (reoxygenation), the cell will undergo apoptosis. If, however, the energy production cannot be resumed, the cell will die by necrosis (Saikumar et al., 1998). It is therefore thought that oxygen deprivation induces apoptotic cell death, and not necrosis, as long as oxygen levels return to normal within a given time period. In addition it has been shown that postischemic reperfusion leads to an overproduction of reactive oxygen species (ROS), which induce apoptosis in mammals (Szeto, 2008). The brain's high oxygen consumption and low levels of antioxidants makes it particularly vulnerable to oxidative damage compared to other organs (Hemmen and Zivin, 2007). ROS is therefore to be regarded as a key factor involved in the induction of anoxic cell death.

In the present study there was no change in the prevalence of apoptosis after 7 days of exposure to anoxia compared to the normoxic control group. During anoxia, the crucian carp needs to conserve energy to prolong the anoxic survival time. Cell death by apoptosis is an energy demanding process (Kerr et al., 1972), so to upregulate apoptosis during the anoxic period would be energetically unfavorable. However, our results do not show any down-regulation of apoptosis either. A possible reason for this is that during anoxia, the crucian carp can produce enough ATP to uphold normal cellular ATP levels and during anoxic periods, several processes in the crucian carp brain is maintained at the normoxic levels. A normal rate of protein synthesis is, for instance, upheld in the crucian carp brain during anoxia (Smith

1996), and a wide range of genes involved in neurotransmission show stable expression during anoxia compared to normoxic conditions (Ellefsen et al., 2008, Ellefsen et al., 2009).

After the first 24 h of reoxygenation following 7 days of anoxia there was an increase in the prevalence of apoptotic cell death compared to both anoxic and normoxic conditions. This result strikingly resembles the effect of an ischemic episode on the mammalian hippocampus, where reperfusion accelerates the rate of cell death (Li et al., 2007). The majority of cell death occurring during reperfusion after brief ischemia is believed to be caused by apoptosis and not necrosis (Pagnussat et al., 2007). Recently Stensløkken et al. (2010) demonstrated a change in the expression levels of heat shock proteins (HSP) during anoxia. The main role of HSPs during episodes of stress (e.g. anoxia) is to preserve protein function, and thus limit cellular damage (Nishi et al., 1993). HSP70, for instance, protects cells against both apoptotic and necrotic cell death (Giffard and Yenari, 2004). A 10 fold increase in the expression of HSP70 mRNA was shown as a response to anoxia in crucian carp at 13 °C, while expression was maintained at a high level before, during and after anoxia at 8 °C (Stensløkken et al., 2010). An increased level of HSP70 mRNA has also been observed during ischemia and reperfusion in mammalian hippocampus CA1 neurons (Nishi et al., 1993). This elevation of HSP70 declined in surviving neurons, but remained high in those that were destined to die (Truettner et al., 2009). It is thus possible that HSP70 functions to suppress apoptosis during anoxia in the crucian carp. The maintained high HSP70 mRNA expression seen during reoxygenation in anoxic crucian carp kept at 8 °C (Stensløkken et al., 2010) could have a protective effect against apoptosis, but in our study apoptosis still increased during reoxygenation. One possible explanation for this could be an increase in ROS formation. ROS levels are known to increase within minutes of reperfusion in mammals, and these molecules can damage cellular components, including proteins and membrane lipids (Niizuma et al., 2010, Traystman et al., 1991). In a study done on goldfish exposed to anoxia at 20 °C, ROS damage (measured as lipid peroxidation) was significantly increased in the brain 14 h after reoxygenation (Lushchak et al., 2001). Though ROS formation can be expected to be higher at 20 °C than at the temperature of the current experiment (9 °C), ROS are still potential candidates for causing apoptosis in the crucian carp brain during reoxygenation.

In conclusion, this study demonstrates that there is no change in apoptosis in the crucian carp telencephalon after 7 days of anoxia, but an additional day of reoxygenation after anoxia leads to increased prevalence of apoptotic nuclei. This shows that the crucian carp is

able to maintain apoptosis at an unchanged level during anoxia, and that there is either some degree of delayed damage to the brain caused by the anoxic conditions, or damage caused by the restoration of oxygen, possibly induced by ROS. There are similarities to the situation in mammals, where the incidence of apoptosis is low during the anoxic period due to a lack of energy, while upon reperfusion there is a large increase in the apoptotic rate, which may be caused by damage attained during anoxia or from the oxygen induced production of ROS.

## References


Bickler, P. E. & Buck, L. T. 1998. Adaptations of vertebrate neurons to hypoxia and anoxia: Maintaining critical Ca2+ concentrations. *Journal of Experimental Biology,* 201, 1141-1152.

Blazka, P. 1958. The anaerobic metabolism of fish. *Physiological zoology.,* 31, 117-128.

Broglio, C., Gomez, A., Duran, E., Ocana, F. M., Jimenez-Moya, F., Rodriguez, F. & Salas, C. 2005. Hallmarks of a common forebrain vertebrate plan: Specialized pallial areas for spatial, temporal and emotional memory in actinopterygian fish. *Brain Research Bulletin,* 66, 277-281.

Broglio, C., Rodriguez, F., Gomez, A., Arias, J. L. & Salas, C. 2010. Selective involvement of the goldfish lateral pallium in spatial memory. *Behavioural Brain Research,* 210, 191-201.

Broughton, B. R. S., Reutens, D. C. & Sobey, C. G. 2009. Apoptotic Mechanisms After Cerebral Ischemia. *Stroke,* 40, E331-E339.

Brunelle, J. K. & Chandel, N. S. 2002. Oxygen deprivation induced cell death: An update. *Apoptosis,* 7, 475-482.

CharriautMarlangue, C. & BenAri, Y. 1995. A cautionary note on the use of the TUNEL stain to determine apoptosis. *Neuroreport,* 7, 61-64.

Chowdhury, I., Tharakan, B. & Bhat, G. K. 2006. Current concepts in apoptosis: The physiological suicide program revisited. *Cellular & Molecular Biology Letters,* 11, 506-525.

Chu, D., Qiu, J. X., Grafe, M., Fabian, R., Kent, T. A., Rassin, D., Nesic, O., Werrbach-Perez, K. & Perez-Polo, R. 2002. Delayed cell death signaling in traumatized central nervous system: Hypoxia. *Neurochemical Research,* 27, 97-106.

Danielisova, V., Gottlieb, M., Nemethova, M., Kravcukova, P., Domorakova, I., Mechirova, E. & Burda, J. 2009. Bradykinin postconditioning protects pyramidal CA1 neurons against delayed neuronal death in rat hippocampus. *Cellular and Molecular Neurobiology,* 29, 871-878.

Deshpande, J., Bergstedt, K., Linden, T., Kalimo, H. & Wieloch, T. 1992. Ultrastructural-changes in the hippocampal Ca1-region following transient cerebral-ischemia - Evidence against programmed cell-death. *Experimental Brain Research,* 88, 91-105.

dos Santos, N. M. S., do Vale, A., Reis, M. I. R. & Silva, M. T. 2008. Fish and apoptosis: Molecules and pathways. *Current Pharmaceutical Design,* 14, 148-169.

Du, C., Hu, R., Csernansky, C. A., Hsu, C. Y. & Choi, D. W. 1996. Very delayed infarction after mild focal cerebral ischemia: A role for apoptosis? *Journal of Cerebral Blood Flow and Metabolism,* 16, 195-201.

Ellefsen, S., Sandvik, G. K., Larsen, H. K., Stensløkken, K. O., Hov, D. A. S., Kristensen, T. A. & Nilsson, G. E. 2008. Expression of genes involved in excitatory neurotransmission in anoxic crucian carp (Carassius carassius) brain. *Physiological Genomics,* 35, 5-17.

Ellefsen, S., Stensløkken, K. O., Fagernes, C. E., Kristensen, T. A. & Nilsson, G. E. 2009. Expression of genes involved in GABAergic neurotransmission in anoxic crucian carp brain (Carassius carassius). *Physiological Genomics,* 36, 61-68.

Elmore, S. 2007. Apoptosis: A review of programmed cell death. *Toxicologic Pathology,* 35, 495-516.

Erickson, G. F. 1997. Defining apoptosis: Players and systems. *Journal of the Society for Gynecologic Investigation,* 4, 219-228.

Gadian, D. G., Aicardi, J., Watkins, K. E., Porter, D. A., Mishkin, M. & Vargha-Khadem, F. 2000. Developmental amnesia associated with early hypoxic-ischaemic injury. *Brain,* 123, 499-507.

Gavrieli, Y., Sherman, Y. & Bensasson, S. A. 1992. Identification of programmed cell-death insitu via specific labeling of nuclear-DNA fragmentation *Journal of Cell Biology,* 119, 493-501.

Giffard, R. G. & Yenari, M. A. 2004. Many mechanisms for Hsp70 protection from cerebral ischemia. *Journal of Neurosurgical Anesthesiology,* 16, 53-61.

Graslkraupp, B., Ruttkaynedecky, B., Koudelka, H., Bukowska, K., Bursch, W. & Schultehermann, R. 1995. In-situ detection of fragmented DNA (Tunel assay) fails to discriminate among apoptosis, necrosis, and autolytic cell-death - A cautionary note. *Hepatology,* 21, 1465-1468.

Hacker, G. 2000. The morphology of apoptosis. *Cell and Tissue Research,* 301, 5-17.

Hemmen, T. M. & Zivin, J. A. 2007. Molecular Mechanisms of Ischemic Brain Disease. In: Stephen, G. W. (Ed.) *Molecular Neurology.* San Diego, Academic Press.

Hodges, H., Sowinski, P., Fleming, P., Kershaw, T. R., Sinden, J. D., Meldrum, B. S. & Gray, J. A. 1996. Contrasting effects of fetal CA1 and CA3 hippocampal grafts on deficits in spatial learning and working memory induced by global cerebral ischaemia in rats. *Neuroscience,* 72, 959-988.

Holopainen, I. E. 2005. Organotypic hippocampal slice cultures: A model system to study basic cellular and molecular mechanisms of neuronal cell death, neuroprotection, and synaptic plasticity. *Neurochemical Research,* 30, 1521-1528.

Holopainen, I. J., Hyvarinen, H. & Piironen, J. 1986. Anaerobic wintering of crucian carp (Carassius-carassius L).2. Metabolic products. *Comparative Biochemistry and Physiology a-Physiology,* 83, 239-242.

Huang, Y. J. & Lu, K. S. 2001. TUNEL staining and electron microscopy studies of apoptotic changes in the guinea pig vallate taste cells after unilateral glossopharyngeal denervation. *Anatomy and Embryology,* 204, 493-501.

Huppertz, B., Frank, H. G. & Kaufmann, P. 1999. The apoptosis cascade - morphological and immunohistochemical methods for its visualization. *Anatomy and Embryology,* 200, 1-18.

Hyvarinen, H., Holopainen, I. J. & Piironen, J. 1985. Anaerobic wintering of crucian carp (Carassius-carassius L) .1. Annual dynamics of glycogen reserves in nature. *Comparative Biochemistry and Physiology a-Physiology,* 82, 797-803.

Johansson, D., Nilsson, G. E. & Doving, K. B. 1997. Anoxic depression of light-evoked potentials in retina and optic tectum of crucian carp. *Neuroscience Letters,* 237, 73-76.

Johansson, D., Nilsson, G. E. & Tornblom, E. 1995. Effects of anoxia on energy-metabolism in crucian carp brain-slices studied with microcalorimetry. *Journal of Experimental Biology,* 198, 853-859.

Johnston, I. A. & Bernard, L. M. 1983. Utilization of the ethanol pathway in carp following exposure to anoxia. *Journal of Experimental Biology,* 104, 73-78.

Kerr, J. F. R., Wyllie, A. H. & Currie, A. R. 1972. Apoptosis - Basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer,* 26, 239-257.

Kirino, T. 1982. Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Research,* 239, 57-69.

Krumschnabel, G. & Podrabsky, J. E. 2009. Fish as model systems for the study of vertebrate apoptosis. *Apoptosis,* 14, 1-21.

Lawen, A. 2003. Apoptosis - an introduction. *Bioessays,* 25, 888-896.

Leblond, J. & Krnjevic, K. 1989. Hypoxic changes in hippocampal-neurons. *Journal of Neurophysiology,* 62, 1-14.

Li, D. D., Shao, Z. H., Vanden Hoek, T. L. & Brorson, J. R. 2007. Reperfusion accelerates acute neuronal death induced by simulated ischemia. *Experimental Neurology,* 206, 280-287.

Lipton, P. 1999. Ischemic cell death in brain neurons. *Physiological Reviews,* 79, 1431-1568.

Love, S., Barber, R., Srinivasan, A. & Wilcock, G. K. 2000. Activation of caspase-3 in permanent and transient brain ischaemia in man. *Neuroreport,* 11, 2495-2499.

Lushchak, V. I., Lushchak, L. P., Mota, A. A. & Hermes-Lima, M. 2001. Oxidative stress and antioxidant defenses in goldfish Carassius auratus during anoxia and reoxygenation. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology,* 280, R100-R107.

Lutz, P. L. & Nilsson, G. E. 2004. Vertebrate brains at the pilot light. *Respiratory Physiology & Neurobiology,* 141, 285-296.

Martin-Villalba, A., Herr, I., Jeremias, I., Hahne, M., Brandt, R., Vogel, J., Schenkel, J., Herdegen, T. & Debatin, K. M. 1999. CD95 ligand (Fas-L/APO-1L) and tumor necrosis factor-related apoptosis-inducing ligand mediate ischemia-induced apoptosis in neurons. *Journal of Neuroscience,* 19, 3809-3817.

McClintock, D. S., Santore, M. T., Lee, V. Y., Brunelle, J., Budinger, G. R. S., Zong, W. X., Thompson, C. B., Hay, N. & Chandel, N. S. 2002. Bcl-2 family members and functional electron transport chain regulate oxygen deprivation-induced cell death. *Molecular and Cellular Biology,* 22, 94-104.

Mizuno, T. A. & Ohtsuka, T. 2009. Quantitative study of apoptotic cells in the goldfish retina. *Zoological Science,* 26, 157-162.

Nakka, V. P., Gusain, A., Mehta, S. L. & Raghubir, R. 2008. Molecular mechanisms of apoptosis in cerebral ischemia: Multiple neuroprotective opportunities. *Molecular Neurobiology,* 37, 7-38.

Negoescu, A., Lorimier, P., LabatMoleur, F., Drouet, C., Robert, C., Guillermet, C., Brambilla, C. & Brambilla, E. 1996. In situ apoptotic cell labeling by the TUNEL method: Improvement and evaluation on cell preparations. *Journal of Histochemistry & Cytochemistry,* 44, 959-968.

Niizuma, K., Yoshioka, H., Chen, H., Kim, G. S., Jung, J. E., Katsu, M., Okami, N. & Chan, P. H. 2010. Mitochondrial and apoptotic neuronal death signaling pathways in cerebral ischemia. *Biochimica Et Biophysica Acta-Molecular Basis of Disease,* 1802, 92-99.

Nikonenko, A. G., Radenovic, L., Andjus, P. R. & Skibo, G. G. 2009. Structural features of ischemic damage in the hippocampus. *Anatomical Record-Advances in Integrative Anatomy and Evolutionary Biology,* 292, 1914-1921.

Nilsson, G. E. 1988. A comparative-study of aldehyde dehydrogenase and alcohol-dehydrogenase activiries in crucian carp and 3 other vertebrates - apparent adaptations to ethanol-production. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology,* 158, 479-485.

Nilsson, G. E. 1989. Effects of anoxia on serotonin metabolism in crucian carp brain. *Journal of Experimental Biology,* 141, 419-428.

Nilsson, G. E. 1990. 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. *Journal of Experimental Biology,* 150, 295-320.

Nilsson, G. E. 2001. Surviving anoxia with the brain turned on. *News in Physiological Sciences,* 16, 217-221.

Nilsson, G. E. & Lutz, P. L. 2004. Anoxia tolerant brains. *Journal of Cerebral Blood Flow and Metabolism,* 24, 475-486.

Nilsson, G. E. & Renshaw, G. M. C. 2004. Hypoxic survival strategies in two fishes: extreme anoxia tolerance in the North European crucian carp and natural hypoxic preconditioning in a coral-reef shark. *Journal of Experimental Biology,* 207, 3131-3139.

Nishi, S., Taki, W., Uemura, Y., Higashi, T., Kikuchi, H., Kudoh, H., Satoh, M. & Nagata, K. 1993. Ischemic tolerance due to the induction of Hsp70 in a rat ischemic recirculation model. *Brain Research,* 615, 281-288.

Nitatori, T., Sato, N., Waguri, S., Karasawa, Y., Araki, H., Shibanai, K., Kominami, E. & Uchiyama, Y. 1995. Delayed neuronal death in the Ca1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *Journal of Neuroscience,* 15, 1001-1011.

Pagnussat, A. D., Faccioni-Heuser, M. C., Netto, C. A. & Achaval, M. 2007. An ultrastructural study of cell death in the CA1 pyramidal field of the hippocapmus in rats submitted to transient global ischemia followed by reperfusion. *Journal of Anatomy,* 211, 589-599.

Rosenbaum, D. M., Gupta, G., D'Amore, J., Singh, M., Weidenheim, K., Zhang, H. & Kessler, J. A. 2000. Fas (CD95/APO-1) plays a role in the pathophysiology of focal cerebral ischemia. *Journal of Neuroscience Research,* 61, 686-692.

Rosenblum, W. I. 1997. Histopathologic clues to the pathways of neuronal death following ischemia/hypoxia. *Journal of Neurotrauma,* 14, 313-326.

Roy, M. & Sapolsky, R. 1999. Neuronal apoptosis in acute necrotic insults: why is this subject such a mess? *Trends in Neurosciences,* 22, 419-422.

Saikumar, P., Dong, Z., Weinberg, J. M. & Venkatachalam, M. A. 1998. Mechanisms of cell death in hypoxia/reoxygenation injury. *Oncogene,* 17, 3341-3349.

Saito, K. & Watanabe, S. 2006. Deficits in acquisition of spatial learning after dorsomedial telencephalon lesions in goldfish. *Behavioural Brain Research,* 172, 187-194.

Shoubridge, E. A. & Hochachka, P. W. 1980. Ethanol - Novel end product of vertebrate anaerobic metabolism. *Science,* 209, 308-309.

Soutschek, J. & Zupanc, G. K. H. 1996. Apoptosis in the cerebellum of adult teleost fish, Apteronotus leptorhynchus. *Developmental Brain Research,* 97, 279-286.

Spencer, R. L. & Bland, S. T. 2007. Hippocampal Neurons. In: George Finkassociate Editors:Bruce, M., Kloet, E. R. D., Robert, R., George, C., Andrew, S., Noel, R., *et al.* (Eds.) *Encyclopedia of Stress.* New York, Academic Press.

Stensløkken, K. O., Ellefsen, S., Larsen, H. K., Vaage, J. & Nilsson, G. E. 2010. Expression of heat shock proteins in anoxic crucian carp (Carassius carassius): support for cold as a preparatory cue for anoxia. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology,* 298, R1499-R1508.

Stensløkken, K. O., Ellefsen, S., Larsen, H. K., Vaage, J. & Nilsson, G. E. 2010. Expression of heat shock proteins in anoxic crucian carp (Carassius carassius): support for cold as a preparatory cue for anoxia. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology,* 298, R1499-R1508.

Surh, C. D. & Sprent, J. 1994. T-cell apoptosis detection in-situ during positive and negavtive selection in the thymus. *Nature,* 372, 100-103.

Suzue, T., Wu, G. B. & Furukawa, T. 1987. High susceptibility to hypoxia of afferent synaptic transmission in the goldfish sacculus. *Journal of Neurophysiology,* 58, 1066-1079.

Szeto, H. H. 2008. Mitochondria-targeted cytoprotective peptides for ischemia-reperfusion injury. *Antioxidants & Redox Signaling,* 10, 601-619.

Taoufik, E. & Probert, L. 2008. Ischemic neuronal damage. *Current Pharmaceutical Design,* 14, 3565-3573.

Traystman, R. J., Kirsch, J. R. & Koehler, R. C. 1991. Oxygen Radical Mechanisms Of Brain Injury Following Ischemia Ans Reperfusion. *Journal of Applied Physiology,* 71, 1185-1195.

Truettner, J. S., Hu, K., Liu, C. L., Dietrich, W. D. & Hu, B. R. 2009. Subcellular stress response and induction of molecular chaperones and folding proteins after transient global ischemia in rats. *Brain Research,* 1249, 9-18.

Ucker, D. S. 1991. Death by suicide - One way to go in mammalian cellular-development. *New Biologist,* 3, 103-109.

Ultsch, G. R. & Jackson, D. C. 1982. Long-term submergence at 3-degrees-C of the turtle Chrysemys-Picta-Bellii in normoxic and severly hypoxic water .3. Effects of changes in ambient PO-2 and subsequent air breathing. *Journal of Experimental Biology,* 97, 87-99.

Van Waversveld, J., Addink, A. D. F. & Van Den Thillart, G. 1989. Simultaneous direct and indirect calorimetry in normoxic and anoxic goldfish. *Journal of Experimental Biology,* 142, 325-336.

Vargas, J. P., Lopez, J. C. & Portavella, M. 2009. What are the functions of fish brain pallium? *Brain Research Bulletin,* 79, 436-440.

Vornanen, M. 1994. Seasonal adaptation of crucian carp (Carassius-Carassius L) heart - Glycogen stores and lactate-dehydrogenase activity. *Canadian Journal of Zoology-Revue Canadienne De Zoologie,* 72, 433-442.

Vornanen, M., Stecyk, J. A. W. & Nilsson, G. E. 2009. The anoxia-tolerant crucian carp (Carassius carassius L.). *Fish Physiology,* 27, 397-441.

Wang, W., Redecker, C., Bidmon, H. J. & Witte, O. W. 2004. Delayed neuronal death and damage of GDNF family receptors in CA1 following focal cerebral ischemia. *Brain Research,* 1023, 92-101.

Wolvekamp, M. C. J., Darby, I. A. & Fuller, P. J. 1998. Cautionary note on the use of end-labelling DNA fragments for detection of apoptosis. *Pathology,* 30, 267-271.

Zemke, D., Smith, J. L., Reeves, M. J. & Majid, A. 2004. Ischemia and ischemic tolerence in the brain: an overview. *Neurotoxicology,* 25, 895-904.

Zhang, A., Wu, Y., Lai, H. W. L. & Yew, D. T. 2004. Apoptosis - A brief review. *Neuroembryology,* 3, 47-59.

Zhang, F., Li, C., Wang, R., Han, D., Zhang, Q. G., Zhou, C., Yu, H. M. & Zhang, G. Y. 2007. Activation of GABA receptors attenuates neuronal apoptosis through inhibiting the tyrosine phosphorylation of NR2A by Src after cerebral ischemia and reperfusion. *Neuroscience,* 150, 938-949.

Zupanc, G. K. H. 2009. Towards brain repair: Insights from teleost fish. *Seminars in Cell & Developmental Biology,* 20, 683-690.

Zupanc, G. K. H., Kompass, K. S., Horschke, I., Ott, R. & Schwarz, H. 1998. Apoptosis after injuries in the cerebellum of adult teleost fish. *Experimental Neurology,* 152, 221-230.

Zupanc, G. K. H. & Zupanc, M. M. 2006. New neurons for the injured brain: mechanisms of neuronal regeneration in adult teleost fish. *Regenerative Medicine,* 1, 207-216.