Gene expression in anoxic crucian carp brain

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kjæreste kjæreste,

du min beste venn -

ligg tett inntil meg,

hvis jeg hadde bare -

en sang igjen,

handlet den om deg

- Ole Paus

Preface

There are times in a life that take ages to pass, times that go by so slowly they seem like forever.

Then, times turn. They become 6 years of passing from youth to grown-up. They become 6 years of going from taught to being teacher. They become 6 years of passing by, and an exit, and an entrance.

Dear Göran. You have, through fun times, thoughtfulness, beer and song, become a close friend. But, most of all, you have, through faith, guidance, discussion and writing, shown me the path to scientific excellence. It has been a great journey. Hopefully, I can rise to some of the heights you made me see.

Dear Tom. Not once did you turn me away when I came knocking, always helpful and smiling. I particularly appreciated your encouragements. They were invaluable.

The Physiology Programme has been a perfect place for intellectual growth, but it has also been a good place for friend growth. Thanks to Øyvind J. for giving me a molecular push. Thanks to Kåre-Olav for fruitful collaborations, both inside and outside rush hours. You are a true friend. Thanks to Guro, Helene, Dag Are, Miriam, Jonas and Cathrine. The guidance was truly mutual. Thanks to Christina for mental sparring and friendship. Thanks to Tove and Ida B for happy office- and lab-hours. Thanks to Erik for fish'n friendchip, Øyvind for hunting in lowlands, Jørund for scientific trigger-pushing, Stine for smiles along a paralleled crusade, Jonathan and Aga for refreshing the last year in Oslo, and to Kristin, Victoria, Harald, and everyone else that made the Programme such a pleasant, though challenging setting.

Thanks to colleagues at Lillehammer University College. You have been very supportive and patient.

Life is nothing without outside-academia experiences. To my dear friends Egil and Dag Are: Blindern will never be the same without BAB. Thanks to Kjetil, Anne Hege, Bent, Guro, *et al.*. Real friends are hard to find. Thanks to my family. In the end, you are all that matters. Thanks to my family-in-law. Days at Trønnes bring peace to mind.

Family is life.

Dear Astri Olea. You are the rain that brings water, and the sunshine that gives life. In your presence, every second is the breeding of another. In your absence, every second is the killing.

Dear Mali. You are the heart.

You are the blood. You are the courage. You are the loyalty and the unconditional. You made the long hours longer, and you were my road back home. Vanished are the years of spring. Our summer is here.

Time was our sacrifice. A life-time together is our award.

Stian Ellefsen

Lillehammer, March 2008

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Abstract

Crucian carp, *Carassius carassius*, survives days to months without oxygen (anoxia). Still, during anoxia, it needs to keep the energy expenditure low, particularly in the brain, which has a high rate of ATP use mainly related to neuronal activity. We hypothesized that the anoxic crucian carp brain reduces its ATP use by suppressing neuronal excitability, and that this is reflected by the expression of genes involved in excitatory and inhibitory neurotransmission.

Real-time RT PCR has become a dominating technique for analyses of gene expression. It enables large-scale, hypothesis-driven analyses of gene expression, and should be well-suited for studies in anoxic crucian carp. However, so far, the use of real-time RT PCR has been limited by the lack of a proper procedure for data normalization, with existing procedures depending on the assumption that internal control genes show constitutive expression and do not vary between experimental groups. This is a particular problem in experiments involving severe physiological stress, such as anoxia, where the expression of control genes must be expected to change.

Paper 1 reports a novel procedure for normalization of real-time RT PCR data, using an external RNA control gene (mw2060). It is the first to report the addition of an external RNA to tissue on a per-unit-weight basis. The procedure was demonstrated to be suitable for normalization of real-time RT PCR data in crucian carp heart and brain, and provided more accurate normalization than internal RNA control genes. For example, in anoxic hearts, β-actin failed to detect a 2.5-fold increase in the expression of the stress-response gene HSC70.

Papers 2 and 3 use the real-time RT PCR procedure to investigate the effects of 1 and 7 days of anoxia on the expression of 29 genes involved in excitatory glutamatergic neurotransmission and 22 genes involved in inhibitory GABAergic neurotransmission, respectively. In general, paper 2 talks against profound neural depression caused by reduced expression of excitatory ion channels in anoxic crucian carp brains. Still, the NMDA receptor-subunits (NR) showed expression patterns that could mediate reduced neuronal excitability. Primarily, the NR2 subunit expression was dominated by NR2B and NR2D, which resembles that seen in hypoxia-tolerant neonatal rats, but also, the expression of NR1, NR2C and NR3A decreased during anoxia, which suggests a reduced number of functional NMDA receptors.

Paper 3 indicates that the GABAergic system in the crucian carp brain is dominated by extrasynaptic components. While the expression of $GABA_A$-receptors subunit was dominated by α_4, α_6, and δ subunits, all of which are located to extrasynaptic sites in mammalian brains and respond to elevations in extracellular levels of GABA by showing tonic activity-patterns, the

expression of GABA transporters was dominated by GAT2 and GAT3, which also show extrasynaptic location in mammals. The majority of the investigated genes were largely unaltered by anoxia, but the expression of GAT2 (a and b) and GAT3 was reduced by up to 80%. This suggests reduced GABA transport in the anoxic crucian carp brain, which may explain the previously reported elevation in extracellular GABA levels, and could underlie the previously observed GABAergic inhibition of anoxic metabolic rate.

List of papers

Paper 1

Improved normalization of real-time reverse transcriptase polymerase chain reaction data using an external RNA control

Anal. Biochem., in press, doi:10.1016/j.ab.2008.01.028.

Ellefsen S., Stensløkken K.-O., Sandvik G. K., Kristensen T. A., Nilsson G. E.

Paper 2

Expression of genes involved in excitatory neurotransmission in anoxic crucian carp brain (*Carassius carassius*)

Submitted

Ellefsen S., Sandvik G. K., Larsen H. K., Stensløkken K.-O., Hov D. A. S., Kristensen T. A., Nilsson G. E.

Paper 3

Expression of genes involved in GABAergic neurotransmission in anoxic crucian carp brain (*Carassius Carassius*)

Submitted

Ellefsen S., Stensløkken K.-O., Fagernes C. E., Kristensen T. A., Nilsson G. E.

Abbreviations

AMPA – alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR – AMPA receptor

ATP – adenosine triphophate

BDNF – Brain-derived neurotrophic factor

Ca_V – voltage-gated calcium channel

Cc – *Carassius carassius*

CLSI – Clinical and Laboratory Standards Institute

CREB1 – cAMP response element-binding protein 1

Cp – crossing point

Dr – *Danio rerio*

E – priming efficiency

EAAT – excitatory amino acid transporter

ERCC – External RNA Control Consortium

GABA – gamma-aminobutyric acid

GABARAP – $GABA_A$-receptor associated proteins

GABAT – GABA aminotransferase

GAD – glutamate decarboxylase

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

GAT – GABA transporter

GluR – AMPA-receptor subunit

HSC70 – heat shock cognate 70

HSP30 – heat shock protein 30

KCC – K^+/Cl^- co-transporter

mw2060 – *Microcystis cf. wesenbergi* 2060 bp, the external RNA control gene

Na_V – voltage-gated sodium channel

NMDA – N-methyl-D-aspartic acid

NMDAR – NMDA receptor

NR – NMDA-receptor subunit

PCR – polymerase chain reaction

Rn – *Rattus norvegicus*

RT PCR – reverse transcriptase PCR

Tn – *Tetraodon nigroviridis*

Tr – *Takifugu rubripes*

TrkB – BDNF receptor

Introduction

Anoxia tolerance

The mechanisms for handling oxygen deprivation vary across the vertebrate lineage, often as a consequence of different needs in different habitats. For example, whereas mammals get their oxygen from air, at or close to sea level, and hardly experience fluctuations in oxygen concentrations, fish get their oxygen from water and frequently experience variations in oxygen concentrations (Nikinmaa and Rees, 2005). Aquatic oxygen levels often vary with water depth, time a day, and time a year (Bickler and Buck, 2007). The physical properties of water (compared to air), including a low oxygen concentration and a slow oxygen diffusion rate, make aquatic hypoxia a common phenomenon (Nilsson and Lutz, 2004; Nikinmaa and Rees, 2005). Still, while hypoxia tolerance is widespread, anoxia tolerance is a rare trait also among aquatic vertebrates. However, a few species have evolved to survive a total lack of oxygen for long periods of time. Fish of the genus *Carassius* and freshwater turtles of the genera *Chrysemys* and *Trachemys* can tolerate days to months of anoxia, depending on temperature (Nilsson and Lutz, 2004; Bickler and Buck, 2007). This allows them to survive wintertime anoxia in small ice-covered lakes in the northern hemisphere.

Vertebrate cells are dependent on continuous supply of energy in the form of adenosine triphosphate (ATP). Since they cannot store energy in this form, ATP must be continuously synthesized from compounds such as glycogen, proteins and fat throughout life. This process is most efficiently carried out in the presence of oxygen, through aerobic ATP synthesis, which for glucose yields approximately 29 moles of ATP per mole of substrate (Martin, 2003). However, ATP can also be synthesized in the absence of oxygen (anoxia). This is done by anaerobic glycolysis, but since the metabolic end-product of this process (often lactate) still contains a lot of chemical energy, it only yields about 10% of the amount of ATP produced by aerobic metabolism (Hochachka and Somero, 2002). Vertebrate cells that experience periods of anoxia are thus faced with a critical challenge: they have to account for the reduced yield of ATP synthesis, or die.

Tolerating anoxia

Increased ATP production or decreased ATP consumption? To survive anoxia the balance between ATP production and ATP consumption needs to be maintained (Lutz and Nilsson, 1997; Bickler and Buck, 2007). This can either be achieved by increasing the rate of anaerobic/glycolytic ATP

production, or by decreasing the rate of metabolic ATP consumption, or by a combination of them both. To solely rely on increased ATP production during anoxia would result in high rates of fuel consumption, which would lead to rapid depletions of fuel stores. Moreover, since anaerobic ATP production usually results in production of lactic acid, it would give acidification of cellular environments and eventually cell death. On the other hand, to solely rely on decreased ATP consumption would put the organism in a defenceless comatose-like condition, and would possibly prolong the anoxic period by impairing the ability to respond to external stimuli and move to oxygenated water.

The anoxia-tolerant crucian carp (*Carassius carassius*) shows increased rates of glycolytic ATP production during anoxia (Johansson et al., 1995), but simultaneously displays decreased production of body heat (Van Waversveld et al., 1989), indicating decreased ATP consumption. Anoxia tolerant freshwater turtles, on the other hand, show inhibition rather than enhancement of glycolytic ATP synthesis during anoxia (Kelly and Storey, 1988), and show drastic reductions in metabolic rate (Herbert and Jackson, 1985; Jackson, 2002). The differences between these two apparently contrasting strategies of anoxia survival are further reflected in the anoxic locomotor activity. While crucian carp remain active during anoxia, although at a reduced rate, freshwater turtles become more or less comatose (Lutz and Nilsson, 1997).

The importance of glycogen stores. Since glycogen is the only energy supply during anoxia, large glycogen stores are vital for tolerating prolonged anoxic periods. Indeed, crucian carp and freshwater turtles have extraordinary large glycogen stores. While up to 30% of the normoxic crucian carp liver mass is glycogen (Hyvärinen et al., 1985), 15% of the freshwater turtle liver mass is glycogen (Clark and Miller, 1973; Lutz et al., 2003). The sizes of the liver glycogen store have been shown to correlate with, and thus to limit the length of the anoxia survival in both crucian carp and freshwater turtles (Nilsson, 1990; Warren et al., 2006). Furthermore, these organisms also show large glycogen reserves in other organs, such as brain and heart (Daw et al., 1967; Vornanen, 1994; Lutz and Nilsson, 1997; Vornanen and Paajanen, 2006). The glycogen stores in brain and heart have been suggested to be important for survival during the initial phases of anoxia (Lutz and Nilsson, 1997).

Avoiding lactic acid accumulation. Both crucian carp and freshwater turtles avoid the negative effects of lactic acid during anoxia. Crucian carp avoids lactic acid accumulation by having ethanol as the main anaerobic end-product (Shoubridge and Hochachka, 1980; Van Waarde, 1991). Since ethanol can subsequently diffuse into the surrounding water across the gill surface, waste accumulation is unlikely to be a factor that limits anoxic survival (Nilsson, 1990). Turtles, on the

other hand, reduce the consequences of lactic acid-mediated acidosis by using their bones and shells for buffering (Jackson, 2000a).

The anoxic brain

In most vertebrates, the brain is particularly sensitive to oxygen depletion and even minutes of anoxia result in a fall in brain ATP levels, membrane depolarization, and accumulation of cytosolic Ca^{2+} and cell death (Kristian and Siesjo, 1996; Lipton, 1999; Arundine and Tymianski, 2003; Lutz et al., 2003). The rapidness of these events is primarily related to the high rate of ATP consumption in brain tissue, largely devoted to maintaining ion gradients, which are constantly challenged by ion fluxes through ion channels such as voltage-gated Na^+ and Ca^{2+} channels (Na_Vs and Ca_Vs), and excitatory ligand-gated channels (Erecinska and Silver, 1994).

Anoxia-intolerant vertebrate brains

As a direct consequence of falling ATP levels in neurons during anoxia, the Na^+/K^+-ATPase fails to maintain the ion distribution across cell membranes, resulting in a net efflux of K^+ (Lutz et al., 2003). Initially, the resulting increase in extracellular $[K^+]$ leads to a progressive depolarization, which soon develops into a general depolarization, probably as a result of the opening of Na_Vs and Ca_Vs (Lutz et al., 2003). This, in turn, may reverse the Na^+/Ca^{2+} exchanger, leading to further influx of Ca^{2+} (Stys et al., 1992). Moreover, a reversal of glutamate transporters, induced by the reduced Na^+ gradient, leads to an efflux of excitatory neurotransmitters such as glutamate, spreading the depolarization from neuron to neuron and enhancing it in single neurons (Villmann and Becker, 2007). Thus, as soon as the ATP storage of oxygen-deprived vertebrate brains is depleted, a cascade of events is entered that eventually result in intracellular accumulation of Ca^{2+}. Intracellularly, Ca^{2+} activate proteases, phospholipases and endonucleases, and also induce the formation of free radicals, processes that eventually kill the cells (Lipton, 1999).

One way of reducing the fatality of an anoxic insult in the vertebrate brain would be to hinder the occurrence of neuronal depolarization. This can be achieved by lowering the membrane permeability of depolarizing ions or by reducing the action potential activity (and has collectively been termed “channel arrest”) (Lutz et al., 1985; Hochachka, 1986). Previous efforts to search for protective mechanisms that give decreased neural excitation, or the lack of such, have focused on the neurotransmitter component, and has primarily aimed to develop neuroprotective drugs for clinical use (Conti et al., 2004; Villmann and Becker, 2007). Neurotransmitter systems such as the

excitatory glutamatergic system, but also the inhibitory GABAergic system, have been preferred targets because they play decisive roles during energy deficiency, and can readily be manipulated using pharmacological tools.

Excitatory neurotransmission during anoxia in the anoxic vertebrate brain. In the vertebrate brain, excitatory neurotransmission is most often mediated by glutamate, which opens ion channels that depolarizes cell membranes. During oxygen deprivation, the release of glutamate is accelerated, leading to excessive opening of glutamate receptors such as AMPA receptors (AMPARs) and NMDA receptors (NMDARs). In experimental models, blocking of AMPARs and NMDARs pharmacologically during oxygen or energy deprivation substantially decrease the extent of neuronal cell deaths (Choi and Rothman, 1990; Palmer, 2001; Szenasi and Harsing, 2004). The NMDARs have been in particular focus, being responsible for a large Ca^{2+} influx. By blocking NMDARs in cultured neurons, the occurrence of cell deaths during energy deprivation has been reduced by up to 83-93% (Tai et al., 2001). Furthermore, neuroprotection by blocking excitatory neurotransmission during oxygen deprivation can also be accomplished by inhibiting Na_Vs and Ca_Vs (Fung, 2000; Nikonenko et al., 2005).

In general, excitatory ion channels are made up of one or more protein subunits, depending on the ion-channel family. While NMDARs and AMPARs consist of 4 subunits (Kohr, 2006; Sprengel, 2006), the ion pores of Na_Vs and Ca_Vs consist of a single protein (Catterall et al., 2003a; Catterall et al., 2003b) (see section on key neurotransmitter receptors and ion channels below). Each of these ion-channel families contains a multitude of subunits or variants, and the properties of functional channels can differ substantially between receptors with different subunit composition or between different variants (Catterall, 2000; Perez-Reyes, 2003; Diss et al., 2004; Cull-Candy et al., 2006; Kohr, 2006; Sprengel, 2006). This potential for ion-channel plasticity has been given particular focus in studies of oxygen-deprivation. For example, in the mammalian brain, ischemia/hypoxia results in decreased expression of the AMPAR subunit GluR2 and the Na_V variant $Na_V1.1$ (Pellegrini-Giampietro et al., 1992; Gorter et al., 1997; Yao et al., 2002), and increased expression of the NMDAR subunit NR2C and the Ca_V variant Ca_V3 (Perez-Velazquez and Zhang, 1994; Small et al., 1997; Del Toro et al., 2003). However, it remains unclear whether these changes represent pathophysiological or physiological processes, and their significance has thus been difficult to elucidate. Further, studies of neonatal mammals, which are known to be hypoxia-tolerant compared to their adult counterparts, have shown that the NMDAR subunit composition is dominated by NR2B and NR2D (Monyer et al., 1994). This has been suggested to be vital for their hypoxia tolerance (Bickler et al., 2003; Bickler, 2004).

Changes in the expression of genes for neurotransmitter transporters have also been suggested to play a role in modulating excitatory neurotransmission during oxygen deprivation. For example, the expression of the glutamate transporter EAAT2 has been found to decrease in the ischemic or hypoxic mammalian brain (Douen et al., 2000; Dallas et al., 2007). This could reflect a pathophysiological event, since it will result in a decreased ability to remove extracellular glutamate. However, studies have suggested that it might be important for the neuroprotective effects of hypoxic preconditioning, reducing the occurrence of glutamate-transporter reversal and stalling the accumulation of extracellular glutamate (Douen et al., 2000). [Hypoxic preconditioning is the ability of a limited hypoxic event to reduce the effects of a subsequent more severe hypoxic/ischemic insult]. This reveals a complexity in the mammalian response to oxygen deprivation, and indicates that it is difficult to separate pathophysiology from protective mechanisms.

Inhibitory neurotransmission during anoxia in the anoxic vertebrate brain. In the vertebrate brain, the neurotransmitter GABA is the prime mediator of inhibitory neurotransmission, providing opening of ion channels that act to hyperpolarize (or clamp) the membrane potential. During oxygen deprivation, such hyperpolarization has been suggested as a way to counteract the uncontrolled depolarization brought about by glutamate. Indeed, the neuroprotective potential of GABAergic ion channels has been demonstrated in several models, showing that pharmacological stimulation of GABAergic activity results in reduced cell death during oxygen deprivation (Green et al., 2000; Schwartz-Bloom and Sah, 2001). However, in the oxygen-deprived mammalian brains the GABAergic activity seems to be lowered rather than enhanced, resulting in neuronal hyperexcitability and cell death (Green et al., 2000; Schwartz-Bloom and Sah, 2001). This occurs despite an increase in extracellular GABA levels (Hagberg et al., 1985; Globus et al., 1991).

In vertebrate brains, GABAergic activity can be regulated by altering the levels of GABA in the extracellular space or by altering the GABA affinity and the level of activity of GABA receptors (Farrant and Nusser, 2005). These processes have all been studied in oxygen-deprived mammalian brains. The mechanisms controlling GABA levels include the GABA synthesizing glutamate decarboxylases (GAD65 and GAD67), the GABA degrading GABA aminotransferase (GABAT), and the GABA transport proteins (GAT1-3 and BGT-1) (Martin and Rimvall, 1993; Treiman, 2001; Conti et al., 2004). The affinity and activity of GABA receptors can be altered by modulating their composition and their abundance (see section on key neurotransmitter receptors and ion channels below) (Mody and Pearce, 2004; Farrant and Nusser, 2005). During oxygen- or energy deprivation in mammals, GAD65 and GAD67 show increased expression (Schwarzer and

Sperk, 1995; Esclapez and Houser, 1999; Sperk et al., 2003), presumably increasing the GABA production in an activity-dependent manner (Ramirez and Gutierrez, 2001), and GABA transporters GAT1 and GAT3 show reduced expression (Melone et al., 2003; Sperk et al., 2003; Zhu and Ong, 2004), presumably leading to reduced GABA uptake and increased extracellular GABA levels. Also, subunits of $GABA_A$Rs and $GABA_B$Rs show altered patterns of expression (Sperk et al., 1998; Redecker et al., 2002; Furtinger et al., 2003). However, again it is difficult to make functional interpretations based on the currently available GABA data, because it is difficult to separate pathophysiology from adaptive mechanisms. Moreover, discrepant results are common. For example, some studies on GABA transporters have indicated that the expression of GAT1 and GAT3 increase, rather than decrease, during oxygen deprivation (Hirao et al., 1998; Melone et al., 2003).

Anoxia-tolerant crucian carp and turtle brains

Brains of anoxia-tolerant vertebrates, such as crucian carp and freshwater turtles, handle anoxia very well, and consequently do not exhibit excessive release of glutamate (Nilsson and Lutz, 1991; Hylland and Nilsson, 1999). These brains maintain the balance between ATP production and ATP consumption (Lutz and Nilsson, 1997; Bickler and Buck, 2007). While the crucian carp brain shows a modest 37% decrease in heat production during anoxia, paralleled by a 2.4 increase in glycolytic rate (Johansson et al., 1995), the turtle brain shows a more dramatic 85-90% decrease in ATP turnover, paralleled by a decrease in glycolytic rate (Lutz et al., 1984; Kelly and Storey, 1988; Jackson, 2000b). Direct evidence for neuronal depression has been found in both species. Crucian carp neural responses to light and sound are strongly depressed in anoxic crucian carp (Suzue et al., 1987; Fay and Ream, 1992), and EEG recordings from anoxic turtle brains have demonstrated a nearly complete suppression of electric activity (Fernandes et al., 1997).

Neuronal depression: reduced excitatory ability or increased inhibitory tone? Studies have suggested a role for reduced activities of excitatory ion channels in the neuronal depression observed in anoxic freshwater turtles. For example, in anoxic turtle neurons, AMPARs show 60% reductions in evoked peak currents and 51% reductions in firing rates (Pamenter et al., 2008). Moreover, NMDARs show 50-65% reductions in opening probability and 67% reductions in Ca^{2+}-current amplitude (Buck and Bickler, 1998; Bickler et al., 2000). Also, studies of gene expression have suggested a suppression of excitatory ion channels, and while the expression of the NMDAR-subunit NR1 has been shown to decrease by 60% during anoxia (Bickler et al., 2000), the abundance of voltage-gated Na^+ channels (Na_Vs) has been shown to fall by 42% (Perez-Pinzon et

al., 1992). Studies on crucian carp, however, have so far failed to demonstrate a role for excitatory ion channels in the anoxic neuronal depression. For example, anoxic crucian carp brains show unchanged membrane permeability of K^+ and Ca^{2+} (Johansson and Nilsson, 1995; Nilsson, 2001).

Inhibitory neurotransmission, on the other hand, seems to provide neuronal depression during anoxia in both crucian carp and turtle brains. In crucian carp, the extracellular level of GABA was doubled after 6 h of anoxia at 10°C, and tissue levels of GABA was increased 5-fold after 17 days of anoxia at 8°C (Nilsson, 1990; Hylland and Nilsson, 1999). Moreover, pharmacological inhibition of GABAergic neurotransmission resulted in a 3-fold increase in whole-body metabolic rates during anoxia (Nilsson, 1992), effects that were not seen in normoxic individuals (Nilsson, 1992). In anoxic turtle brains, the extracellular levels of GABA were increased 80-fold after 6 hours of anoxia (Nilsson and Lutz, 1991). Also in turtles, pharmacological inhibition of the GABAergic response seems to increase the levels of neuronal activity, resulting in increased levels of extracellular glutamate (Thompson et al., 2007).

Although the density of $GABA_A$ receptors has been shown to increase in the turtle brain during anoxia (Lutz and Leone-Kabler, 1995), arguably providing increased GABAergic inhibition, few studies have addressed the roles of the different GABAergic components, such as GABA-metabolizing enzymes, GABA transport and GABA receptors, in the anoxia tolerance of crucian carp and freshwater turtles.

Fig. 1 summarizes key adaptations shown by crucian carp and turtles during anoxia.

Key neurotransmitter receptors and ion channels

AMPA receptors

AMPARs are ionotropic glutamate receptors that consist of four subunits (denoted GluR). In mammals, four different subunits (GluR1-4) have been identified (Sprengel, 2006), and in zebrafish, 8 different subunits have been identified (GluR1a,b-4a,b). Functional AMPARs are either homo- or hetero-tetrameric (Sprengel, 2006).

AMPARs are permeable to Na^+ and K^+, and most often also to Ca^{2+}, and are vital for excitatory synaptic neurotransmission (Sprengel, 2006). Their activity is influenced by several factors, in particular the subunit composition, but also post-transcriptional and post-translational modifications and auxiliary proteins (Sprengel, 2006).

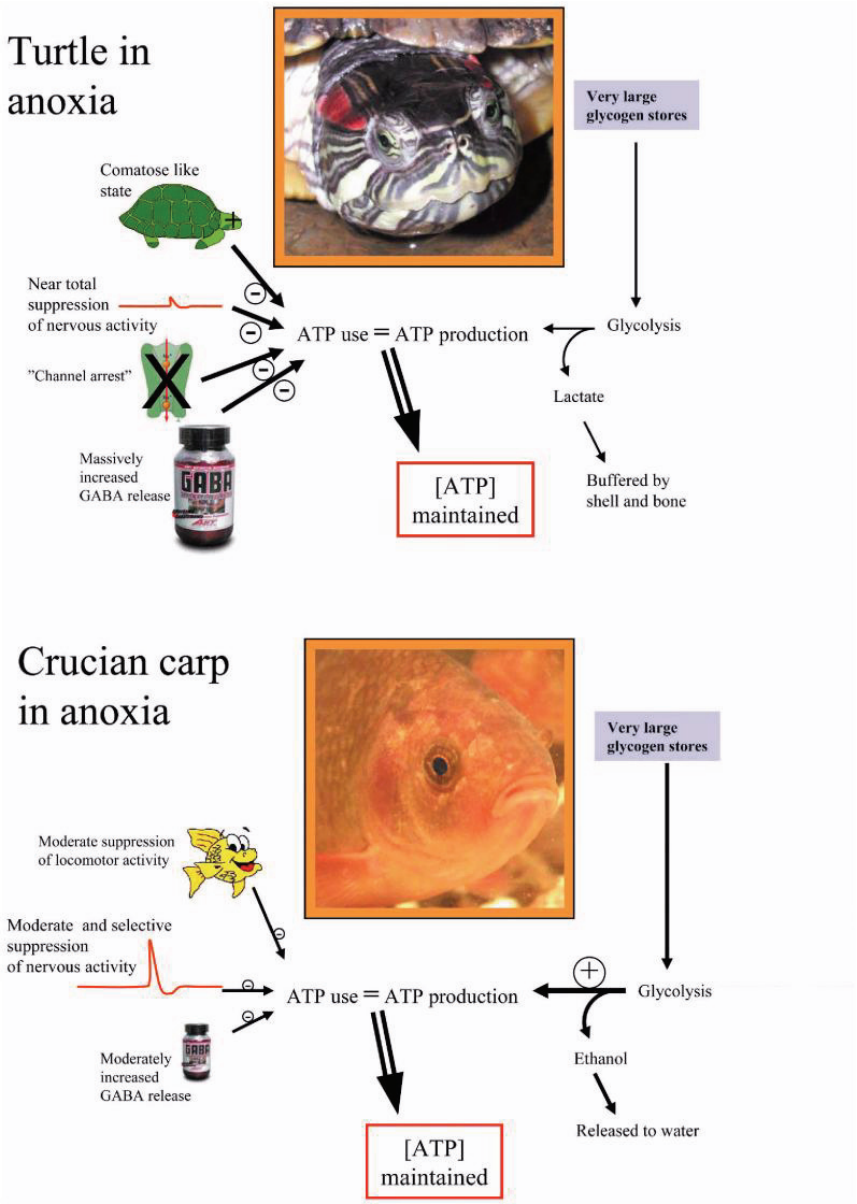

Fig. 1. The key to anoxic survival in both turtles and crucian carp is their ability to maintain brain tissue ATP levels during anoxia. This can only be done by matching ATP use with glycolytic ATP production. The turtle strategy is to reduce ATP use as much as possible by a near total suppression of nervous activity mediated by reduced ion permeability of membranes and release of inhibitory factors like GABA. Hereby glycolytic lactate production can be reduced to levels that can be tolerated through buffering. The crucian carp remains active in anoxia and is able to match ATP use with ATP production through up-regulated glycolysis. Here, the main role of its relatively modest metabolic depression is probably to save on the glycogen stores, since it avoids lactate poisoning and acidosis by turning lactate into ethanol. Modified from Nilsson and Lutz 2004.

NMDA receptors

NMDARs are ionotropic glutamate receptors that consist of four subunits (denoted NR). In mammals, 7 different subunits (NR1, NR2A-D, NR3A-B) have been identified. Functional receptors consist of two NR1 subunits and two NR2 subunits (Kohr, 2006). The NR3 subunits seem to act as modulators of the NR1/NR2 receptor (Dingledine et al., 1999), but NR3A has also been indicated to co-assemble with NR1 and form glycine-sensitive excitatory receptors (Chatterton et al., 2002).

NMDARs are permeable to cations such as Na^+, K^+ and Ca^{2+}, and are vital for excitatory neurotransmission (Dingledine et al., 1999). In contrast to the opening of AMPARs, glutamate-binding is not sufficient for the opening of NMDARs, and glycine-binding and membrane depolarization are also required. In mammals, both NR1 and NR2 subunits are obligatory components of NMDARs, though the NR2 composition can be varied and is a vital source for variation in electrophysiological properties, providing developmental and regional diversity (Dingledine et al., 1999). In addition, the properties of NMDARs will be affected by post-transcriptional and post-translational modifications and auxiliary proteins (Wenthold et al., 2003).

GABA receptors

GABA receptors fall into two groups; ionotropic $GABA_A$Rs and metabotropic $GABA_B$Rs (Pierce et al., 2002; Farrant and Nusser, 2005).

$GABA_A$Rs are ionotropic GABA receptors that consist of five subunits (Farrant and Nusser, 2005). The subunit-diversity is large, and in mammals 19 different subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π and ρ_{1-3}) have been identified. Functional receptors typically consist of 2α-2β-1γ.

$GABA_A$Rs are permeable to anions, primarily Cl^- and HCO_3^-, and represent the most important mediators of inhibitory neurotransmission in the mammalian brain. The properties of $GABA_A$Rs are primarily decided by their subunit composition, and in particular by the α-subunit composition, but also by post-transcriptional and post-translational modifications and auxiliary proteins (Farrant and Nusser, 2005). Most importantly, $GABA_A$Rs can be classified into two functionally different types, based on the subunit composition: 1) phasic synaptic receptors, responsible for synaptic GABAergic inhibition (e.g. $\alpha_1\beta_2\gamma_2$) (Farrant and Nusser, 2005), and 2) tonic extrasynaptic receptors, responsible for extrasynaptic inhibition caused by ambient levels of GABA (e.g $\alpha_{4/6}\beta_2\delta$) (Farrant and Nusser, 2005).

$GABA_BRs$ are metabotropic GABA receptors that consist of two subunits. In mammals, 2 different subunits (G_B1 and G_B2) have been identified (Pierce et al., 2002). Functional receptors contain both (Pierce et al., 2002).

$GABA_BRs$ are G-protein coupled, and thus mediate their response through second messengers (Bettler and Tiao, 2006). Despite the limited number of $GABA_BR$ subunits, rendering subunit plasticity impossible, considerable variation in receptor function can be achieved by post-transcriptional and post-translational modifications and auxiliary proteins (Bettler and Tiao, 2006).

Voltage-gated ion channels

The opening of voltage-gated Na^+ and Ca^{2+} channels (Na_Vs and Ca_Vs) is controlled by the membrane potential, and their ion pores typically consist of a single α protein, associated with auxiliary β proteins (Catterall et al., 2003a; Catterall et al., 2003b). In mammals, 10 α proteins have been identified for each of the two families (Catterall et al., 2003a; Catterall et al., 2003b).

In neurons, Na_Vs are involved in propagating action potentials, while Ca_Vs are required for neurotransmitter release. The different α subunits of the Na_V- and Ca_Vs-families provide ion channels with different electrophysiological properties (Catterall et al., 2003a; Catterall et al., 2003b).

Molecular aspects of hypoxia tolerance

Genomes and phenotypes

The phenotype of an organism is defined by its collective expression of functional proteins; the proteome (Hack, 2004). It is plastic, and can be altered in response to physiological challenges such as anoxia (Nikinmaa and Rees, 2005; Cossins et al., 2006). An organism's ability to tolerate such challenges is ultimately determined by its genomic composition, and specific genomic sequences are thus inevitably linked to the anoxia tolerance of crucian carp and freshwater turtles.

On the protein level, anoxia tolerance is likely to appear both as constitutively expressed traits, making the animal prepared for anoxic periods, and inducible traits, being turned on during anoxic events. Characterizing these adaptations can both clarify how these animals have evolved to tolerate anoxia, and provide insight into why most vertebrates fail to survive anoxia (Pritchard, 2002; Nilsson and Lutz, 2004).

Proteins or mRNA

Phenotypic traits are often studied by assessing the functional status of specific proteins. Organisms control protein function either by regulating protein abundance (protein synthesis/degradation), or by regulating activities of already existing proteins (Blackstock and Weir, 1999; Zhu et al., 2001). Thus, ideally, all studies of protein function should assess protein activity. However, such measurements are difficult to achieve, and large-scale investigations of protein function are often limited to measuring protein abundance, either directly or by quantifying mRNA.

At present, preferred techniques for studying protein abundance are immunoassays such as western blotting, which addresses the abundance of individual proteins by using monoclonal or polyclonal antibodies (Kingsmore, 2006). These techniques allow quantification of protein levels as well as post-transcriptional modifications (Olive, 2004), and can thus be used to provide insight into protein function. However, immunoassays have several limitations and may not always be the best alternative. In the present context, which involves analyses of gene expression in fish, a major problem with immunoassays is that they depend on antibodies. Available antibodies are usually made against mammalian proteins and may not recognize homologous proteins in distantly related vertebrates such as fish. This problem is especially worrying for analyses of proteins that are members of protein families. Such families often contain closely related proteins, and the antibodies may not be able to distinguish the different family members and may therefore result in erroneous estimates of protein abundance. In addition, immunoassays are impeded by the lack of proper methods for controlling the specificity of the protein-antibody binding, particularly when dealing with proteins that show differences in molecular weight between vertebrate species.

The limitations of immunoassays make way for approaches that quantify protein abundance indirectly by assessing mRNA, such as real-time RT PCR.

Real-time RT PCR

Real-time RT PCR has become a preferred tool for studies of gene expression in biological and medical research (Arya et al., 2005; Nolan et al., 2006). It can be used for all organisms, and its only requirement is that the target gene sequence is known. It is easily accessible, extremely sensitive, and accurate (Nolan et al., 2006). The specificity of real-time RT PCR assays can readily be controlled by cloning and sequencing and the technique can be used for quantification of closely related genes/paralogs. In addition, it has a relatively large potential for rapid, high through-put performance and is capable of mapping traits such as differential splicing.

Still, when using real-time RT PCR to study gene expression it is necessary to keep in mind that protein activity is not always reflected by mRNA abundance, as proteins may be regulated at other levels, such as translation or degradation (Greenbaum et al., 2003). In the context of the present study, which assesses proteins such as ion-channel subunits, it is fortunate that proteins that act as components of multi-protein complexes have been found to correlate well with mRNA levels (Jansen et al., 2002). The properties of such protein complexes are probably decided by the stoichiometric availability of their subunits/components (Jansen et al., 2002; Greenbaum et al., 2003).

Despite the potential of real-time RT PCR in quantifying specific mRNA species, the technique has its limitations (Nolan et al., 2006). The gravest of these concerns the normalization process, where a critical premise is the presence of stably expressed internal RNA control genes (reference genes or "housekeeping genes") (Bustin et al., 2005; Huggett et al., 2005; Nolan et al., 2006). If this requirement is not fulfilled, then measuring the expression of target genes in relation to internal control genes will be inaccurate, giving erroneous conclusions (Tricarico et al., 2002; Bas et al., 2004; Dheda et al., 2005). Since the expression of traditional control genes, such as β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and cyclophilins, have frequently been shown to change in response to experimental treatment (Bustin, 2000; Sturzenbaum and Kille, 2001; Huggett et al., 2005; Wong and Medrano, 2005), numerous studies have aimed at developing alternative approaches for normalization. These include normalization to factors calculated from multiple internal RNA control genes (the geNorm procedure) (Vandesompele et al., 2002), normalization to total RNA concentrations (Bustin, 2000) and normalization to externally added, alien RNA controls (Toscani et al., 1987; Freeman et al., 1999; Shibata et al., 1999; Smith et al., 2003). Despite the efforts, neither have become standard procedures (Nolan et al., 2006).

Thus, the need for a proper procedure for normalization of real-time RT PCR data is urgent. This need is particularly apparent in experiments involving fluctuations in key environmental factors such as salinity, temperature, nutrient supply and oxygen, where the organism is likely to undergo large-scale physiological adaptations, leading to changes in the expression of internal RNA control genes.

Aims of the thesis

The overall aim of this thesis was to examine the effects of anoxia on various components of excitatory and inhibitory neurotransmission in crucian carp brain. These systems are likely to control the activity of the brain during anoxia, and therefore to contribute to anoxic survival by depressing ATP use. This was done by measuring the expression of relevant genes in brain tissue of normoxic and anoxic crucian carp using real-time RT PCR.

Since anoxia is a severe physiological challenge that is likely to cause wide-ranging changes in gene expression, a method for normalization of gene expression that is independent of internal control genes had to be developed. Moreover, since the crucian carp genome has not been sequenced, most genes had to be cloned from crucian carp brain.

- In *paper 1* the aim was to develop a procedure for normalization of real-time RT PCR data using an external RNA control gene.

- In *paper 2* the aim was to investigate the expression of genes involved in excitatory neurotransmission in the normoxic and anoxic crucian carp brain.

- In *paper 3* the aim was to investigate the expression of genes involved in GABAergic neurotransmission in the normoxic and anoxic crucian carp brain.

Methodological considerations

Standardization of the external RNA control protocol

While developing the external RNA control procedure, the need for a standardized protocol became gradually more apparent, and only after having standardized every step could proper normalization of real-time RT PCR data be achieved. Since then, the procedure has been successfully applied in numerous experiments, some of which are presented in this thesis. Standardization at the levels of RNA extraction, cDNA synthesis and real-time RT PCR was found to be particularly important.

RNA extraction

RNA extractions were performed using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). This procedure is based on the single-step RNA isolation protocol published by Chomczynski and Sacchi (1987; 2006), and was chosen for several reasons. Firstly, it enables rapid extraction of high quality RNA from minute amounts of tissue. Secondly, it does not involve columns or other physical barriers or tools, such as magnetic beads, which might lead to loss of RNA. Thus, the RNA yield is likely to reflect the amount of RNA in the tissue samples, enabling comparison of RNA content between experimental animals. Thirdly, the amounts of tissue extracted can readily be scaled up or down during the TRIzol procedure. This permitted the smallest available tissue sample to limit the extraction (see further comments below).

The RNA control gene, mw2060, was added to the tissue samples prior to homogenization on a per-mg basis. Since the external RNA control gene was not a part of the internal environment of the cells, its usefulness relied on the RNA extractions to show similar efficiencies. To ensure this, we developed a standardized TRIzol protocol, where RNA was extracted from equal amounts of tissue within experiments. Also, to avoid systematic errors introduced by sample processing, a few premises had to be followed. Primarily, all samples had to be handled without intermission, using the same aliquot of mw2060. Moreover, within each experiment, the samples were handled in a particular order, wherein the handling order of the oxygen exposure groups N7, A1, A7 and A7N3/A7N7 continuously shifted.

Reverse transcription

Experimental variation in the real-time RT PCR performance can often be ascribed to variation in the reverse transcription of RNA (the cDNA synthesis) (Stahlberg et al., 2004a; Stahlberg et al., 2004b). To minimize the negative influence of such variation, standardization of the protocol was required, both within experiments and between experiments. This included using the same type of reverse transcriptase in all experiments, using the same technique for priming in all experiments, performing all cDNA syntheses of an experiment simultaneously, and performing all cDNA syntheses in duplicates. [Note: in paper 1, one of the experiments included only one cDNA synthesis. To account for this, a larger number of experimental animals were used.]

Real-time RT PCR

Careful design of the real-time RT PCR step is another critical determinant of data quality. In the current studies this included keeping pipetting volumes above 5 µl and running real-time RT PCR reactions for each gene and each cDNA synthesis in duplicates.

Notably, primer optimization was performed indirectly by testing three primer pairs for each gene and using the primer pair that worked best with the outlined real time RT PCR protocol (primer concentration of 100 nM and annealing temperature of 60° C). This was evaluated to be a preferred alternative to changing the protocol to suit single primer pairs.

Why not microarrays?

Microarray is another technique that is frequently used for studies of mRNA expression. In contrast to real-time RT PCR, which typically assesses one gene at a time, microarray experiments has the potential to assess thousands of different genes (Gracey et al., 2004). Thus, microarray seems better suited for large-scale studies. However, it has its limitations, and was not preferred in these studies. Primarily, it is confined by the selection of genes that are included on the array, often being rather arbitrary. For example, of the 54 genes investigated in papers 1, 2 and 3 only 14 genes were found in carpBASE 5.0 (http://legr.liv.ac.uk/searchDB/ search_carpbase_5_0.php), a database based on microarray-related clones produced by The Laboratory for Environmental Gene Regulation (LEGR), University of Liverpool, and containing over 22500 annotated carp sequences. Also, intrinsic constraints of the microarray technique, such as unspecific hybridization, prohibit analyses of closely related genes and splice variants, features that can easily be resolved using real-time RT PCR. This concern would have been particularly grave for crucian carp, for which no species-specific microarray exists.

Synopsis of results

Paper 1

This paper reports a novel procedure for normalization of real-time RT PCR data using an external RNA control gene. It is the first to report the addition of an external RNA to tissue on a per-unit-weight basis. Its accuracy, suitability and usefulness in experiments involving a severe physiological challenge are demonstrated. The expression of the internal RNA control genes (reference genes) β-actin, cyclophilin A and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was investigated in brain and heart of normoxic and anoxic crucian carp (*Carassius carassius*). Their expression differed significantly between experimental groups, especially in heart. Consequently, these internal RNA control genes were unsuitable for normalization of real-time RT PCR data. For example, in anoxic hearts, β-actin and geNorm (the geometric average of β-actin, cyclophilin A and GAPDH) failed to detect a 2.5-fold increase in the expression of the stress-response gene HSC70. Further, the need for an optimized and standardized external RNA control protocol is demonstrated and discussed. Collectively, the data suggest that the normalization of real time RT PCR data is considerably improved by adding an external RNA control to the samples.

Paper 2

This paper uses the real-time RT PCR procedure described in paper 1 to investigate the effects of 1 and 7 days of anoxia (12ºC) on the expression of 29 genes involved in excitatory neurotransmission in crucian carp brain. The genes include 8 AMPA-receptor subunits (GluR1a,b-4a,b), 6 NMDA-receptor subunits (NR1, NR2A-D and NR3A), 7 voltage-gated sodium and calcium channels ($Na_V1.1$, 1.3 and 1.6, and $Ca_V2.1$, 2.3, 3.1 and 3.2), 4 glutamate transporters (EAAT2a, 2b, 3a and 3b) and 4 genes involved in NMDAR-mediated neuroplasticity (CREB-1, BDNF and the BDNF receptors TrkB1 and TrkB2). The majority of the subunits and variants of the ion-channel families showed expression profiles (indicative of relative subunit composition) similar to those observed in the normal mammalian brain, and showed remarkably stable expression during anoxia. This indicates that the genes have coinciding functions in crucian carp and mammals, and that the excitatory abilities of the crucian carp brain are retained during anoxia. Although the data generally talk against a role for reduced capacities of excitatory neurotransmission in neural depression ("channel arrest"), NMDA receptor-subunit (NR) expression showed changes that could mediate reduced neural excitability. Primarily, the NR2 subunit expression, which was dominated by NR2B

and NR2D, resembled that seen in hypoxia-tolerant neonatal rats, and a decreased expression of NR1, NR2C and NR3A during anoxia indicated a reduced number of functional NMDA receptors. The full-length sequence of crucian carp NR1 mRNA is also reported. A novel NR1 splice cassette, introducing an N-glycosylation site into the extracellular S1S2 domain was found.

Table 1 summarizes the expression of genes involved in excitatory neurotransmission in the anoxic and reoxygenated crucian carp brain.

Table 1. mRNA levels of genes involved in excitatory neurotransmission in the anoxic and reoxygenated crucian carp brain. Data sets were normalized to the external RNA control mw2060, and are compared to the control group N7. A1 = anoxia 1 day; A7 = anoxia 7 days; R4 = anoxia 7 days followed by reoxygenation 4 days. ↓ or ↑ = P<0.05 (one-way ANOVA followed by Tukey Kramer's post test).

Gene	A1	A7	R4
GluR1a	–	–	–
GluR1b	–	–	–
GluR2a	–	–	–
GluR2b	↑	–	–
GluR3a	–	–	–
GluR3b	–	–	–
GluR4a	–	–	–
GluR4b	–	–	–
NR1	–	↓	↓
NR2A	–	–	–
NR2B	–	–	↓
NR2C	↓	↓	↓
NR2D	–	–	–
NR3A	–	↓	–
$Na_V1.1$	–	↑	–
$Na_V1.3$	–	–	–
$Na_V1.6$	–	–	–
$Ca_V2.1$	–	–	–
$Ca_V2.3$	–	–	–
$Ca_V3.1$	–	↑	–
$Ca_V3.2$	–	–	–
EAAT2a	–	–	–
EAAT2b	–	–	↓
EAAT3a	–	–	–
EAAT3b	–	–	–
CREB-1	–	–	–
BDNF	–	–	–
TrkB1	–	–	↓
TrkB2	–	–	↓

Paper 3

This paper uses the real-time RT PCR procedure described in paper 1 to investigate the effects of 1 and 7 days of anoxia (8ºC) on the expression of 22 genes involved in GABAergic neurotransmission in crucian carp brain. The genes include 9 $GABA_A$-receptor subunits ($G_A\alpha_{1-6}$, β_2, δ and γ_2), 3 $GABA_B$-receptor subunits (G_B1a-1b and G_B2), 3 GABAergic enzymes (GAD65 and GAD67, GABAT), 4 GABA transporters (GAT1, 2a-b and 3), 2 $GABA_A$-receptor associated proteins (GABARAP 1 and 2) and a K^+-Cl^- co-transporter (KCC2). The $GABA_A$ receptor of the crucian carp brain was dominated by the extrasynaptic and tonic α_4, α_6, and δ subunits, and the GABA transporter expression was dominated by the extrasynaptic GAT2 and GAT3. The expression of GAT2 (a and b) and GAT3 decreased by up to 80% during anoxia, which could be a mechanism behind the increased extracellular GABA levels observed in the anoxic crucian carp brain. However, the GABA receptor expression was also decreased, generally by about 30%, which may function to limit excessive GABAergic inhibition, possibly through negative feed-back.

Table 2 summarizes the expression of genes involved in GABAergic neurotransmission in the anoxic and reoxygenated crucian carp brain.

Table 2. mRNA levels of genes involved in GABAergic neurotransmission in anoxic and reoxygenated crucian carp brain. Data sets were normalized to the external RNA control mw2060, and are compared to the control group N7. A1 = anoxia 1 day; A7 = anoxia 7 days; R7 = anoxia 7 days followed by reoxygenation 7 days. ↓ or ↑ = $P<0.05$ (one-way ANOVA followed by Tukey Kramer's post test).

Gene	A1	A7	R7
$G_A\alpha1$	–	–	–
$G_A\alpha2$	–	–	↓
$G_A\alpha3$	↓	↓	↓
$G_A\alpha4$	–	–	–
$G_A\alpha5$	–	–	–
$G_A\alpha6$	–	↓	–
$G_A\beta2$	–	–	–
$G_A\delta$	–	–	–
$G_A\gamma2$	–	–	–
G_B1a	–	–	–
G_B1b	–	–	–
G_B2	–	–	–
KCC2	–	–	–
GRAP1	–	–	–
GRAP2	–	–	–
GAD65	–	↓	↓
GAD67	–	–	–
GABAT	–	–	–
GAT1	–	–	–
GAT2a	↓	↓	↓
GAT2b	–	↓	–
GAT3	↓	↓	↓

General discussion

Normalization using an external RNA control

A guideline on how to use external RNA controls, published by Warrington et al. in 2006, stated that their utilization should be limited to monitoring technical performance and should not be expected to perform as control genes for particular transcripts. By contrast, the data presented in this thesis suggest that external RNA controls, when properly introduced to samples, are well-suited for normalization of real-time RT PCR data.

To my knowledge, paper 1 is the first study to show that an external RNA control can be introduced to samples on a "per unit weight of tissue" basis prior to homogenization, and then be used for normalization of real-time RT PCR data. Such normalization provides quantification of gene expression on a "per living cell" basis, given that the experimental system contains stable amounts of water and that there is no net loss/gain of cells, assumptions that seem tenable for anoxic crucian carp (Van der Linden et al., 2001; Sollid et al., 2005).

Several findings indicated the suitability and accuracy of the procedure. First, the standard curve showed a close correlation between amounts of external RNA control (mw2060) added during RNA extraction and amounts of mw2060 quantified using real-time RT PCR. Second, the reproducibility of the procedure was demonstrated by mw2060 providing similar gene expression profiles for similar experiments performed at different temperatures and times. Third, the accuracy of the procedure was indicated by comparisons with non-normalized real-time RT PCR data. While the non-normalized internal RNA control gene data showed significant variation between treatment groups, resembling the mw2060-normalized data, the non-normalized mw2060 data did not show variation between groups. Finally, the non-normalized mw2060 data showed small SDs, indicating technical reproducibility.

At the same time as reporting a novel procedure for normalization of real-time RT PCR data, paper 1 underlines the inaccuracy of existing procedures. Both internal RNA control genes and total RNA abundances were unsuitable for normalization of real-time RT PCR data. This failure is in line with other studies (Bustin, 2000; Sturzenbaum and Kille, 2001; Huggett et al., 2005; Wong and Medrano, 2005), and supports the notion that, until now, proper normalization of real-time RT PCR data has been difficult to obtain in studies involving severe physiological challenges.

Future prospects for external RNA controls

The external RNA control procedure has potential beyond the applications demonstrated in papers 1-3. For example, it is likely to enable comparisons of gene expression across different stages of development and across different types of tissues. The only requirement seems to be that the samples show similar RNA-extraction efficiencies. Normally, this should not represent a problem. However, the issue has not been properly studied (Huggett et al., 2005), and particular care should be taken when samples display large compositional differences.

In theory, the procedure also holds the potential to provide comparisons of gene expression between different experiments and laboratories. However, in paper 1, considerable variation was observed in the mw2060-normalized expression of specific genes between experiments, and inter-experimental comparisons may be difficult to achieve. For example, a 33-fold difference was seen in the normoxic expression of β-actin between 8°C and 13°C crucian carp hearts, which were studied at different times. This difference was unlikely to be caused by biological variation alone, and might be an effect of variation in mw2060 performance, probably caused by batch-to-batch variations or partial degeneration of mw2060 during storage.

Can real-time RT PCR be trusted for gene profiling?

In papers 2 and 3, subunit profiling of AMPARs, NMDARs and GABAARs and gene profiling of GABA transporters is presented. These analyses enabled assessment of properties related to subunit and transporter composition, which is a major determinant of function (Conti et al., 2004; Farrant and Nusser, 2005; Kohr, 2006; Sprengel, 2006).

Gene profiling is dependent on accurate and representative data sets, and although papers 1-3 involved extensive standardization of the real-time RT PCR protocol in order to minimize technical variation, errors may have been introduced during reverse transcription and real-time PCR, whereby different genes may have been differentially affected. If this was the case, then gene profiling would have been inadequate. However, this problem seemed unlikely for several reasons. Primarily the majority of the gene families showed expression profiles that correlate well with corresponding profiles from mammalian brains. For example, in resemblance with the mammalian brain, the AMPAR subunit expression of the crucian carp brain was dominated by the GluR2 subunit (Sprengel, 2006), the flop-variant of the GluR2a subunit was dominated by the flip-variant (Sprengel, 2006), and the NR1 expression exceeded the overall NR2 expression (Goebel and Poosch, 1999). Indeed, in normoxic crucian carp brain, the NR1 subunit constituted 71% of the

total expression of NMDAR subunits (NR1+NR2), which resembles the corresponding number from rat (69-87%) (Goebel and Poosch, 1999). Also, differences in the performance of real-time RT PCR were minimized through two steps: 1) for each gene, three primer pairs were tested, whereupon the most efficient primer pair was chosen, and 2) for each primer pair, the priming efficiency was calculated, which means that differences in priming efficiency between real-time RT PCR assays should have been accounted for.

Neurotransmission in anoxic crucian carp

Are crucian carp brains preset to handle anoxia?

A possibility that was considered in papers 2 and 3 is that the crucian carp brain shows gene expression patterns during normoxia that set the stage for anoxia tolerance. Paper 2 indicates that NMDARs of crucian carp neurons have an NR2 composition that leads to relatively low ion conductance, providing increased anoxic tolerance. Paper 3 suggests that the GABAergic system of the crucian carp brain is dominated by extrasynaptic, tonic receptors, which are likely to allow a relatively high inhibitory tonus in response to the sustained elevated levels of extracellular GABA that have been observed in the anoxic crucian carp brain (Hylland and Nilsson, 1999).

The NR2 composition of crucian carp brains was dominated by NR2B and NR2D, with a correspondingly low abundances of NR2A and NR2C (paper 2). Although this is rather different from what is observed in adult rat brains, it resembles what is seen in neonatal rat brains. Interestingly, like crucian carp, neonatal rats display high degrees of hypoxia tolerance. Indeed, this hypoxia tolerance has, at least in part, been attributed to the NR2 composition (Bickler, 2004), and seems to decrease in parallel with the replacement of NR2B and NR2D with NR2A and NR2C seen during maturation (Wang et al., 1995; Dunah et al., 1996; Wenzel et al., 1996; Wenzel et al., 1997). Also, during hypoxia, NMDARs containing the NR2D subunit exhibit reduced ion currents, while those containing the NR2C subunit exhibit increased currents (Bickler et al., 2003), further underlining the potential for subunit composition to mediate anoxia tolerance.

Compared to the GABAergic system of mammalian brains, which is dominated by synaptic components such as the $GABA_AR$ subunits α_1 and γ_2, and the GABA transporter GAT1 (Conti et al., 2004; Farrant and Nusser, 2005), the GABAergic system in crucian carp is dominated by the extrasynaptic tonic $GABA_AR$ subunits α_4, α_6, and δ, and the extrasynaptic GABA transporters GAT2a-b and GAT3. The extrasynaptic $GABA_ARs$ may be essential for responding to the elevated GABA levels found in crucian carp during anoxia (Hylland and Nilsson, 1999). Indeed, in

mammals, such receptors have GABA affinities (EC_{50} values) (Brown et al., 2002; Feng and Macdonald, 2004) that coincides well with the measured GABA levels in the anoxic crucian carp brain (Hylland and Nilsson, 1999). Furthermore, high levels of extrasynaptic GATs are likely to allow close control over extracellular GABA levels.

Adapting to anoxia

The majority of the genes investigated in papers 2 and 3 did not show altered expression in crucian carp brains during anoxia. For example, of the 22 excitatory ion channels and glutamate-receptor subunits investigated in paper 2, only 3 showed altered expression (Table 1), and of the 11 inhibitory GABA-receptor subunits investigated in paper 3, only 2 showed altered expression (Table 2). Thus, anoxic crucian carp largely seems to maintain its ability to respond to neurotransmitters. This gives support to the idea that the crucian carp shows normoxic gene expression patterns that are necessary for anoxic survival. However, the anoxic crucian carp brain also showed changes in the expression of excitatory and GABAergic genes that are likely to contribute to the anoxic tolerance. Arguably, these changes decreased excitatory neurotransmission, either by reducing receptor density or by providing increased inhibitory activity.

Paper 2 reported decreased expression of the NMDAR-subunits NR1 in the crucian carp brain during anoxia, indicating decreased abundance of functional NMDARs (Fukaya et al., 2003), but it also reported decreased expression of NR2C, indicating reduced ion fluxes through the NMDARs that are actually present (Bickler et al., 2003). As previously discussed, the expression of NR2C was relatively low already in normoxic crucian carp, and it seems reasonable to assume that replacing NR2A and NR2C with NR2B and NR2D provide increased tolerance to anoxia.

Paper 3 showed decreased expression of the extrasynaptic GABA transporters GAT2a,b and GAT3 in the crucian carp brains during anoxia (down to 20% of normoxic expression), suggesting that the elevated levels of extracellular GABA observed in anoxic brains (Hylland and Nilsson, 1999) is obtained by impaired GABA transport. This could be a key mechanism behind the observed GABAergic inhibition of anoxic metabolic rate (Nilsson, 1992). Surprisingly, paper 3 also suggested that the expression of $GABA_A$ receptor subunits is slightly reduced in the anoxic crucian carp brain. This could be a negative feed-back response to the elevated GABA levels, and may function to limit the inhibitory tonus during anoxia, thereby allowing an appropriate level of neural activity. Also the reduced expression of the GABA-synthesizing enzyme GAD65 could be such a feed-back response.

Anoxia-tolerant vertebrates as models for medical research

In the human brain, the most common cause of oxygen deprivation is artery occlusion. Such insults are leading causes of human death and disability worldwide (Chang et al., 2007), and has for long been in focus in medical research. Traditional models for these studies have been mice and rats, and the aims have been to map protective or pathophysiological mechanisms, and to develop drugs to enhance or attenuate these mechanisms. However, the use of mammalian models has largely failed to provide drugs that give neuroprotection following acute ischemic strokes in humans (Bickler, 2004). Although numerous efforts have aimed at developing antagonistic drugs against target proteins such as ionotropic glutamate receptors, all attempts have failed to result in clinical neuroprotection (Lizasoain et al., 2006; Villmann and Becker, 2007). Moreover, the use of these antagonists is often associated with severe side effects such as nausea, hallucinations and paranoia (Ginsberg, 2008).

The failures of mammalian models have led to the suggestion that anoxia-tolerant vertebrates, such as crucian carp, may be better suited for mapping neuroprotective pathways (Lutz and Nilsson, 1997; Bickler, 2004); primarily because they tolerate anoxia, and thus represent apparent solutions to the problem, but also because they do not exhibit pathophysiology, which simplifies data analyses. On the negative side, compared to mammals, crucian carp show differences in brain anatomy and brain function compared to mammals. This will limit its applicability in medical research. Still, with regard to neurotransmission, crucian carp expresses more or less the same proteins as mammals (papers 2 and 3), and in the case of the NR1 gene, which was the only one to be full-length cloned in the current studies, all regions coding for key properties were conserved (paper 2). Thus, it seems reasonable to expect that crucian carp will provide a fresh view on the anoxia problem, resulting in ideas for new targets in clinical research.

Paper 3 suggests that the GABAergic system shows a particularly high degree of anoxia-induced plasticity in crucian carp. It is therefore tempting to suggest that manipulation of GABAergic activity is a more effective way of altering neuronal activity than manipulation of glutamatergic activity. This is largely in contrast to the glutamatergic focus that has dominated medical research for the last decades. Furthermore, regarding the role of GABA transporters in regulating GABAergic activity, mammalian studies have centered on GAT1 (Dalby, 2003). However, in crucian carp, GAT1 does not seem to play a role in the anoxia-induced GABAergic plasticity. Instead, GAT2 and GAT3 seem to be key proteins, presumably being responsible for regulating extracellular levels of GABA and thus also to regulate the tonic GABAergic activity (paper 3). Interestingly, this fits well with a recent study on rats, suggesting that GAT3, and not

GAT1, is important for regulating tonic GABAergic activity in hypothalamic neurons (Park et al., 2006).

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