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Neuronal hypoxia tolerance in diving endotherms

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Glossary and abbreviations

Glossary

Aerobic: Transformation of matter and energy that requires oxygen.

Anaerobic: Transformation of matter and energy without uptake of oxygen.

Angiogenesis: The development and growth of capillaries.

Anoxia: Anoxia means 'without oxygen', an extreme form of hypoxia.

Asphyxia: A condition of restricted gas exchange characterized by an increase of blood PCO_2 above and decrease of blood PO_2 below normal values.

Concentration of a gas (%): The concentration of a gas is proportional to the partial pressure (def. below) of the gas, and also to the solubility of the gas in a given substance**.** Thus, the partial pressure of a gas dissolved in two different substances in contact will always be equal, while the concentration of the gas is dependent upon its solubility in the respective substances.

Ectotherm: An animal in which regulation of body temperature depends mainly on behaviorally controlled exchange of heat with the environment.

Endotherm: An animal in which body temperature depends on a high and controlled rate of heat production.

Hypometabolism: Decreased energy metabolism.

Hypoxemia: A state in which the oxygen pressure and/or concentration in arterial and/or venous blood is lower than its normal value at sea level.

Hypoxia: Any state in which the oxygen in the lung, blood and/or tissues is abnormally low compared with that of normal resting man breathing air at sea level.

Ischemia: A decrease in blood supply to a bodily organ, tissue, or part caused by constriction or obstruction of the blood vessels.

Necrosis: Death of cells or tissues through injury or disease, especially in a localized area of the body.

The **partial pressure** or **tension** of a gas in a gas mixture is the fractional concentration of that gas times the total pressure of all gases (i.e. the P_{O2} of atmospheric air is 0.21 x 760 mmHg = 160 mmHg at sea level). A gas will diffuse in the direction of lower partial pressure until the partial pressure is equal between two substances.

Pasteur-effect: The inhibiting effect of oxygen on the process of fermentation.

Q_{10}-effect: The increase or decrease of a reaction rate due to a 10 ^{o}C change in temperature.

Neuronal **shut-down** refers to a general cessation of activity brought about by reduced ion flux ('channel arrest') and most likely suppressed metabolism.

Sources: Farlex free medical dictionary (**www.thefreedictionary.com**); Glossary on respiration and gas exchange (**J. Appl. Physiol. 34, 1973, 549-558**); Glossary of terms for thermal physiology (**J. Thermal Biol. 28, 2003, 75-106**); Human Physiology – The Mechanisms of Body Function**, A. Vander, J. Sherman, D. Luciano, 7th Edition, WCB/McGraw-Hill, U.S.A**.

Abbrevations (in order of appearance)

O_2 - oxygen

ATP – adenosine triphosphate

Pa_{O2}/Pa_{CO2} – arterial partial pressure of O_2/CO_2

Hb – hemoglobin

LDH – lactate dehydrogenase

EEG – electroencephalogram

aCSF – artificial cerebrospinal fluid

SCA/∫SCA – spontaneous cerebellar activity/ integrated spontaneous cerebellar activity

NADH – the reduced form of nicotinamide adenine dinucleotide (NAD)

K_{ATP} – ATP-sensitive K^+ channels

Ngb – neuroglobin

Cyt c – cytochrome oxidase c

GABA – gamma-aminobutyric acid

NMDA – *N*-methyl-D-aspartate

VC – visual cortex

List of papers

This thesis is based on five papers that are referred to in the text by their Roman numerals as follows:

I. L.P. Folkow, J.M. Ramirez, S. Ludvigsen, N. Ramirez, A.S. Blix. Remarkable neuronal hypoxia tolerance in the deep-diving adult hooded seal (*Cystophora cristata*). **Neurosci. Lett. 446 (2008), 147-150**.

II. J.M. Ramirez, L.P. Folkow, S. Ludvigsen, N. Ramirez, A.S. Blix. Spontaneous and persistent activity in thick neocortical slices of the deep-diving hooded seal. Manuscript

III. S. Ludvigsen, L.P. Folkow. Differences in *in vitro* cerebellar neuronal responses to hypoxia in eider ducks, chicken and rats. **J. Comp. Physiol. A 195 (2009), 1021-1030**.

IV. S. Geiseler, S. Ludvigsen, L.P. Folkow. K_{ATP}-channels and their possible contribution to neuronal hypoxia tolerance in the cerebellum of eider ducks (*Somateria mollissima*). Manuscript

V. S. Ludvigsen, L.P. Folkow, E.B. Messelt. Brain capillary density in the deep-diving hooded seal (*Cystophora cristata*). Manuscript

Introduction

Dependence on oxygen

Mammals and birds are endotherms which give them the advantage of having much greater stamina and work output relative to ectotherms. However, endothermy has a high metabolic cost that relies on the oxygen-dependent process of oxidative phosphorylation to produce adenosine triphosphate (ATP) at sufficient rates. This is why oxygen (O_2) is crucial for survival of mammals and birds.

Cellular effects of hypoxia

The ATP demand of most mammalian tissues tends to remain constant even if O_2 supply should decrease, and they will therefore typically experience energy deficiency during hypoxia. In order to compensate for this deficit the tissues have the option to produce ATP anaerobically. This is, however, an unfortunate situation since the amount of ATP produced per mole of substrate during anaerobic metabolism is much lower compared to in oxidative metabolism. To meet metabolic demands substrate consumption has to rise considerably, thereby rapidly depleting local stores of substrate. This is known as the so-called Pasteur effect (Sussman et al., 1980). Furthermore, anaerobic metabolism leads to accumulation of deleterious end-products such as H^+. Depletion of substrate and accumulation of end-products are the two fundamental metabolic problems in dealing with hypoxia (e.g. Hochachka, 1986).

According to the extent of its electrical activity, between 20 to 80% of a neuron's resting metabolic rate is used to maintain a homeostatic intracellular environment (e.g. Boutilier, 2001). This maintenance is performed by ATP-dependent pumping systems such as the Na^+/K^+-ATPases, and these will fail if ATP-levels decline. Failure leads to the cascade of events that are illustrated in figure 1 (Boutilier, 2001); membrane depolarization and an

uncontrolled influx of Ca^{2+} through voltage-gated Ca^{2+} channels ('channel leak'), while collapse of the Na^{+} gradient causes sodium-glutamate cotransporters to eject glutamate into the extracellular space. This triggers glutamate receptors, initiating further Ca^{2+} influx and excitatory injury (excitotoxicity) accelerating the membrane depolarization even more. The rise in intracellular Ca^{2+} activates phospholipases and Ca^{2+}-dependent proteases that result in uncontrolled cellular swelling and, ultimately, in cell necrosis (Boutilier, 2001).

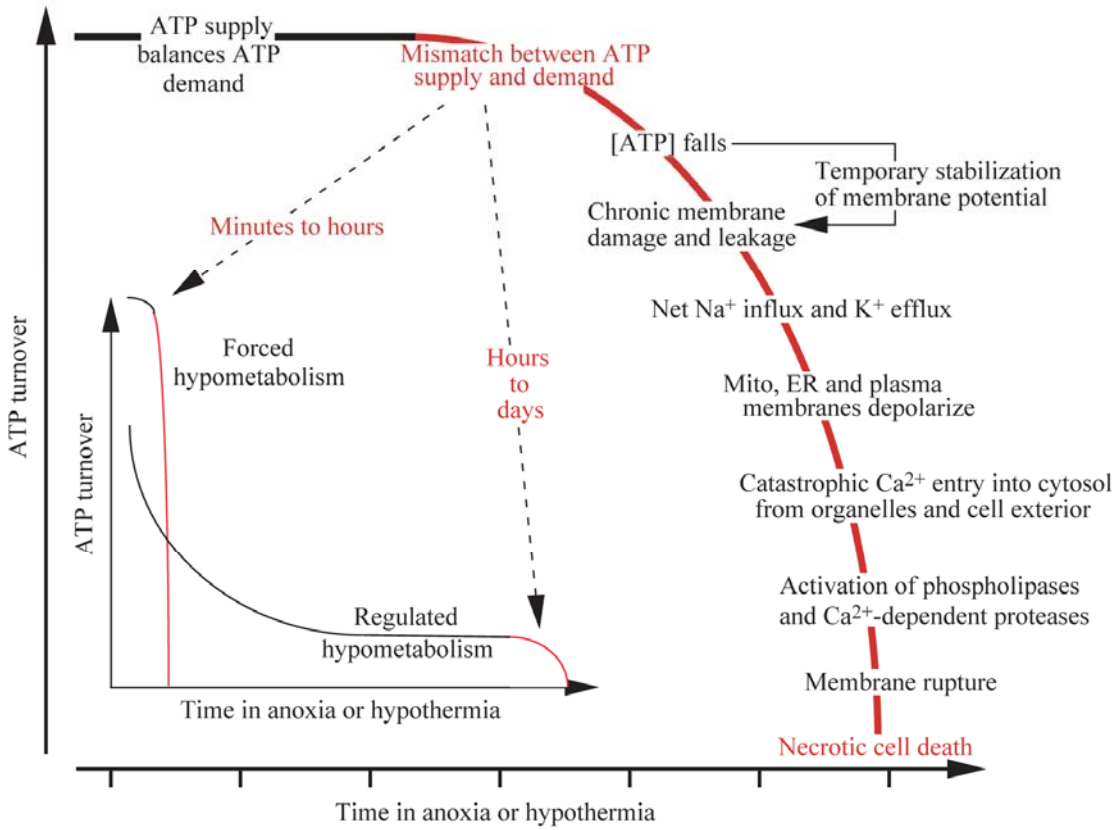

Figure 1: The cascade of events in hypoxia-sensitive cells leading to necrotic cell death that is brought about by a mismatch between ATP supply and demand. The inset shows that a regulated suppression of ATP turnover extends the time to reach the onset of this cascade. From (Boutilier, 2001), reproduced with permission from The Company of Biologists Limited.

Hypoxia tolerance among species is highly variable

The ability to tolerate hypoxia varies greatly between species, some turtles and fish being able to survive anoxia for several weeks (Boutilier, 2001; Nilsson, 2001; Bickler and Buck, 2007). Humans, on the other hand, loose consciousness within seconds when in severe hypoxia (Rossen et al., 1943). Diving endotherms are positioned in between these extremes, apparently being able to withstand severe hypoxia for some time (Scholander, 1940; Hudson and Jones, 1986; Qvist et al., 1986; Stockard et al., 2005; Meir et al., 2009).

Diving endotherms such as seals and ducks are confronted with a basic problem each time they dive, a problem first addressed in detail by Scholander (1940). The problem arises from the fact that these animals have to stop breathing whenever they dive in order to avoid drowning. When they stop breathing, their arterial O_2 tension (Pa_{O2}) starts decreasing and the arterial CO_2 (Pa_{CO2}) tension starts increasing (Scholander, 1940). As such, the longer an animal dives, the more likely it is to face the challenges of hypoxia.

The hooded seal (*Cystophora cristata*) is a record diver relative to its size, normally diving to 300-600 m with dive durations of 5-25 minutes, but some individuals appear to specialize in repetitive deep diving to more than 1,000 m, with durations of up to one hour (Folkow and Blix, 1999). Emperor penguins (*Aptenodytes forsteri*) are able to dive to depths of 500 meters, with durations of more than 15 minutes (Kooyman and Kooyman, 1995). The more modest eider duck (*Somateria mollissima*) has been reported to dive to depths of 60 meters in the field (Cantin et al., 1974), and although there is no record of their maximum dive duration, other ducks tolerate head submersion for more than 10 minutes (Hudson and Jones, 1986; Caputa et al., 1998), which is quite exceptional in light of the fact that the estimated aerobic dive limit (ADL) in eider ducks for shallow foraging dives is a mere 51 seconds (Hawkins et al., 2000). The impressive dive capabilities of both seals and ducks are possible due to several physiological adaptations that are common to all diving birds and

mammals, often referred to as the diving responses. These adaptations have been described extensively elsewhere (Andersen, 1966; Blix and Folkow, 1983; Butler and Jones, 1997; Butler, 2004; Ramirez et al., 2007; Folkow and Blix, 2010). In short, they include an enhanced capacity to store O_2 in blood and skeletal muscles, and an ability to reduce O_2 consumption. Reduced O_2 consumption is achieved in part through redistribution of blood, limiting blood supply to most tissues except particularly hypoxia-sensitive tissues such as heart and brain, thereby delaying onset of hypoxia and allowing the animals to extend their dive time. It has nonetheless been demonstrated that blood O_2 content can drop as low as 10% (Scholander, 1940) and that Pa_{O2} may drop below 20 mmHg (Qvist et al., 1986; Meir et al., 2009) in diving seals. Similarly, Pekin ducks (*Anas platyrhynchos*) have been shown to tolerate a Pa_{O2} of 4kPa (30 mmHg) following forced head submersion (Hudson and Jones, 1986), and air sac P_{O2} in freely diving emperor penguins (*Aptenodytes forsteri*) dropped below 20 mmHg in 42% of all dives recorded by Stockard et al. (2005). Furthermore, according to Kerem and Elsner (1973), the lowest P_{O2} at which reversible brain malfunction appeared in harbor seals (*Phoca vitulina*) performing simulated dives, was 10 mmHg in arterial and 2.5 mmHg in venous blood. This is way below what can be tolerated by non-diving species (Siesjö, 1978; Erecinska and Silver, 2001), and the expert divers tolerate this apparently without ill effects. It seems, therefore, that diving endotherms must have some adaptations that enable their brains to continue to operate even under severely hypoxic conditions.

Neuronal hypoxia tolerance in diving mammals and birds

While the knowledge on how expert divers can endure long-duration dives by delaying the onset of hypoxia is quite extensive (Andersen, 1966; Blix and Folkow, 1983; Butler and Jones, 1997; Butler, 2004; Ramirez et al., 2007; Folkow and Blix, 2010), we know less about how they tolerate hypoxic insult to their central nervous system (CNS). The brain of most endotherms is highly dependent on O_2 and is generally regarded as being very hypoxia sensitive. Despite this, some diving animals seem to repeatedly experience severe hypoxemia (e.g., Qvist et al. 1986, Meir et al. 2009) that inevitably must affect their brain. How is it possible that these animals endure this situation without neuronal damage? With this question in mind, the overall aims of this PhD project were:

1) To investigate if diving mammals and birds have higher intrinsic neuronal hypoxia tolerance than do non-diving mammals and birds.

2) To investigate neuronal mechanisms that may be involved in neuronal hypoxia defences.

3) To investigate if increased brain capillarization may contribute towards the implied enhanced neural hypoxia tolerance.

To accomplish this, models using the deep diving adult hooded seal and the diving eider duck were established. Layer V pyramidal cells from the visual cortex and Purkinje cells from the cerebellum were chosen as targets in these models. Controls (non-divers) were mice (*Mus musculus*), Wistar rats (*Rattus norwegicus*) and domestic chicken (*Gallus gallus domesticus*).

Hypoxemia in deep diving seals

Several seal species have impressive diving capabilities, both expressed as diving depths and durations (e.g. Folkow and Blix, 1999), as well as tolerance to Pa_{O2} values (Scholander, 1940; Qvist et al., 1986) that are lower than those tolerated by non-diving species (Siesjö, 1978; Erecinska and Silver, 2001). There is a possibility that seals cope with such low Pa_{O2} due to enhanced brain blood flow (Blix et al., 1983) and brain capillarization (Kerem and Elsner, 1973; Glezer et al., 1987), but an alternative or additional hypothesis is that neurons of deep diving seals are intrinsically more hypoxia tolerant.

Neuronal hypoxia tolerance in hooded seals vs. mice

The mammalian CNS is notably vulnerable to lack of oxygen (Siesjö, 1978; Lipton, 1999), cortical layer V being among the most vulnerable areas (Brierley and Graham, 1984). However, intracellular recordings of pyramidal cells of cortical layer V from the visual cortex revealed that severely hypoxic hooded seal neurons depolarized at a significantly slower rate than did hypoxic mouse neurons (paper I). Further, the time until hooded seal neurons ceased to discharge was longer than in mouse neurons, and 14% of seal neurons were still active after 60 min in severe hypoxia, which none of the mouse neurons were capable of. Thus, based on the rate of depolarization and time until neurons were unable to discharge, it was concluded that the effect of hypoxia was clearly less pronounced in hooded seal neurons than in adult mouse neurons. Neurons from neonate mice behaved very similarly to seal neurons, both with regard to depolarization rate and ability to continue to discharge in hypoxia. Neural tissue from neonates is known to be more tolerant towards hypoxia than neural tissue from the adult (Singer, 1999), and the fact that the response of the adult hooded seal neurons and the response of neonate mouse neurons were similar, and that the hypoxic responses were studied

under standardized and carefully controlled experimental conditions, shows that hooded seal neurons have an enhanced hypoxic tolerance.

It could be argued that a mouse neuron expectedly would be more sensitive to hypoxia than a seal neuron due to the fact that mass specific metabolic rate increases with decreasing body mass (Kleiber, 1932; Schmidt-Nielsen, 1984). However, West and colleagues (West et al., 2002) demonstrated that the metabolic rate of cultured mammalian cells converge to a single value independent of the size of the mammal of origin, and concluded that the ¼ decrease in metabolism relative to mass seen in cells *in vivo* is a result of constraints in vascular and metabolic transport networks.

Enhanced cerebral hypoxia tolerance has previously been demonstrated in diving mammals in situ (Kerem and Elsner, 1973; Qvist et al., 1986) (figure 2), in that brain function seems largely unaffected at Pa_{O2} below critical values typical of non-diving adult mammals (Erecinska and Silver, 2001). A likely partial explanation for this tolerance is the protective effects of a 2-3 °C brain cooling demonstrated in pinnipeds during diving (Scholander et al., 1942; Odden et al., 1999; Blix et al., 2002), expectedly reducing ATP-consumption via a Q_{10} effect and offering protection from toxic metabolites and oxidative stress (Liu and Yenari, 2007). Another part of the explanation may be the denser brain capillarization reported for seals (Kerem and Elsner, 1973) and dolphins (Glezer et al., 1987), as well as the reported increase in cerebral blood flow in response to increasing P_{CO2} towards the end of dives in seals (Blix et al., 1983). None of these factors could contribute to explain the observed differences between seal and mouse neurons in response to hypoxia (paper I), however, since the experiments were executed with isolated slices that were subject to identical temperatures. Thus, our findings demonstrate differences in neuronal hypoxia tolerance that must be a reflection of intrinsic properties of the neurons themselves.

A **B**

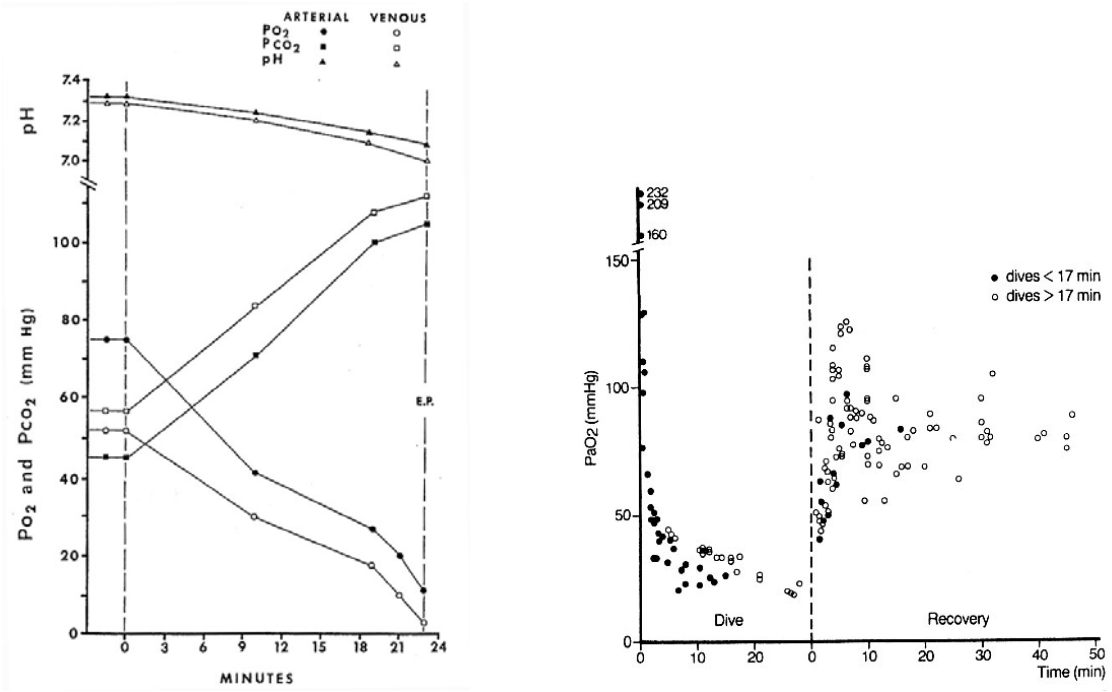

Figure 2: Cerebral hypoxia tolerance demonstrated in diving seals. **A:** Single experiment showing time course of changes in gas tensions and pH of arterial and cerebral-venous blood during diving asphyxia in the harbor seal. From (Kerem and Elsner, 1973), printed with permission from Elsevier. **B:** Changes in arterial O_2 tension (P_{aO2}) during diving and after resurfacing in Weddell seals. From (Qvist et al., 1986), reproduced with permission from APS.

Unusual observations in isolated seal slices

During our electrophysiological experiments it was discovered that thick (680 µm) hooded seal neocortical slices displayed spontaneous activity that could be readily recorded extracellularly (paper II). This is in contrast to the low level of spontaneous activity normally observed in cortical slices of non-diving mammals in standard aCSF (Sanchez-Vives and McCormick, 2000; Shu et al., 2003). Also, slices of the thickness (680 µm) used from the seals are rarely used in studies of less hypoxia-tolerant species since the conditions in their core are severely hypoxic, even during oxygenation (Bingmann and Kolde, 1982; Fujii et al.,

1982; Lipton and Whittingham, 1984; Lipinski and Bingmann, 1986; Fujii, 1990). Indeed, the O_2 tension in the seal brain slices was close to the lower detection limit of our measuring device (0.02-0.6%) at 200 μm depth in normoxia, and despite this, non-periodic long-lasting activity was even maintained for an average of 26 minutes in hypoxia (and in some cases lasted the entire hypoxic exposure of 1 hour). Thus, one reason why we were able to record spontaneous activity in the seal cortical slices is evidently the hypoxia tolerance of hooded seal neurons (paper I).

Cortical and subcortical neuronal networks interact to generate rhythmic patterns of activity at a variety of frequencies (Steriade et al., 1993a; Steriade et al., 1993b; McCormick and Bal, 1997). Previously it was thought that such rhythms can only be studied *in vivo,* and that they result from the interaction of very large networks including subcortical inputs (McCormick and Bal, 1997) since isolated neocortical slabs (a slab is a small perfused volume of an intact brain where afferent and efferent nerve-paths have been cut) *in vivo* (Timofeev et al., 2000) or slices *in vitro* (Dani et al., 2005; Jacobson et al., 2005; Waters and Helmchen, 2006) are largely silent and only display synchronized network activity under certain circumstances. However, the probability of recording large-scale slow oscillating activity *in vivo* with frequency and regularity resembling that generated in the intact cortex increases with increasing slab size (Timofeev et al., 2000). The general silence or very low spontaneous activity of neocortical slices that are studied *in vitro* may therefore be linked to the fact that slices are typically quite thin, in the order of 300-400 μm, and therefore may lack the intact circuitry required for generating and maintaining recurrent activity (paper II). Among the 10 spontaneously active seal brain slices, four slices generated rhythmic population activity involving coordinated activation of multiple units in standard aCSF. Again, this is in contrast to neocortical slices of less hypoxia-tolerant animals that may display intermittent tonic activity (Dani et al., 2005) or rhythmic activity (< 1 Hz) resembling slow wave sleep

(Sanchez-Vives and McCormick, 2000) only after altering the ionic composition of the aCSF. And again, the most likely explanation for these unusual observations is an elevated hypoxia tolerance of the seal neurons, allowing the use of thicker slices than normally used (paper II). This is supported by the fact that the slices generating rhythmic population activity, just as the ones with non-periodic activity, were able to maintain their coordinated activity in hypoxia (>40 minutes). In contrast, loss of coordinated network activity is the first hypoxic effect observed in the neocortex of non-diving mammals (Fleidervish et al., 2001).

Hypoxemia in diving birds

Diving birds (e.g. ducks) survive asphyxia of considerably longer duration than that tolerated by non-diving birds (e.g. chicken) (Bert, 1870; Andersen, 1966; Bryan, Jr. and Jones, 1980). As with diving seals, their ability to tolerate asphyxic hypoxia depends on a set of adaptations that delay the onset of severe hypoxemia. But ultimately P_{aO2} will drop and may leave diving birds in a state of severe hypoxemia. Thus, several diving birds seem to tolerate severe and repeated hypoxic (hypoxemic) insults without ill effects (Blix and Berg, 1974; Hudson and Jones, 1986). Brain cooling in connection with diving probably contributes to this end (Caputa et al., 1998). It has also been suggested that this higher tolerance of the diving species may rely on an enhanced cerebral hypoxic tolerance (figure 3) (e.g., Bryan, Jr. and Jones, 1980; Bickler et al., 1989), as demonstrated in neural tissue of the hooded seal (paper I). This possibility was addressed in paper III.

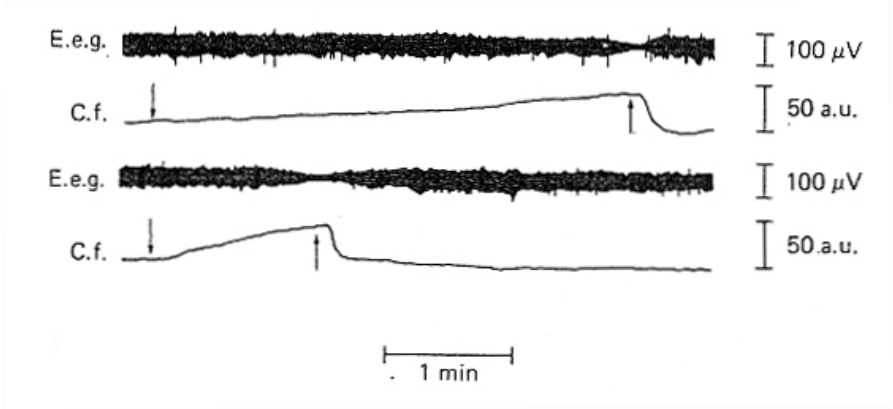

Figure 3: EEG and NADH fluorescence (C.F.) recorded from a duck (upper two traces) and a chicken (bottom two traces) during apnoeic asphyxia, demonstrating the difference in hypoxia tolerance between diving and non-diving birds. Downward pointing arrows indicate beginning of asphyxia and upward arrows indicate the end of asphyxia. The time bar applies to all four traces. From (Bryan, Jr. and Jones, 1980), reproduced with permission from APS.

Inherent hypoxia tolerance of cerebellar neurons from ducks, chicken and rats

In experiments with isolated cerebellar slices from ducks and chicken (and rats), using extracellular recordings in the Purkinje cell layer, all slices displayed rhythmic or non-rhythmic spontaneous cerebellar activity (SCA) in normoxia that could be continuously recorded for > 8 hrs (paper III). Mammalian cerebellar Purkinje neurons, including those of hooded seals (Ludvigsen and Folkow, unpublished observations) discharge spontaneously in slice preparations and as dissociated cells (Llinas and Sugimori, 1980; Wu et al., 1994; Hausser and Clark, 1997; Raman and Bean, 1999; Edgerton and Reinhart, 2003; Akemann and Knöpfel, 2006). However, spontaneous neuronal activity in unmanipulated cerebellar slices or isolated Purkinje neurons obtained from avian species have not previously been reported, suggesting that paper III is the first to report this phenomenon. The observation was predictable, however, given that the cerebellum is highly conserved across vertebrate species (e.g. Sultan and Glickstein, 2007), and that avian cerebellar Purkinje neurons *in vivo* display

spontaneous spiking activity at frequencies that are similar to those described in mammals (Wilson et al., 1974; Belcari et al., 1977).

In comparing hypoxia tolerance in eider duck and chicken cerebellar slices (paper III), no significant differences were found between ducks and chicken in the proportions of slices surviving (i.e. maintaining activity in, or recovering from) hypoxia (95% N_2, 5% CO_2). However, relative levels of integrated spontaneous cerebellar activity ($\int$ SCA) in duck slices were significantly higher than in chicken slices in all surviving slices. Moreover, a significantly higher proportion of duck cerebellar slices survived one hour of severe hypoxia in combination with chemical anoxia (NaCN), and, as with experiments with hypoxia only, the level of $\int$ SCA in duck slices during recovery from chemical anoxia was significantly higher than in identically treated chicken slices. Together, these results led to the conclusion that cerebellar neurons of the eider duck are more tolerant to hypoxia/chemical anoxia than are those of the non-diving chicken. As all experiments were conducted with isolated slices under identical *in vitro* conditions, the observed differences in tolerance to hypoxia/chemical anoxia must arguably be due to different properties inherent to the neural tissue. It is also worth mentioning that similarly high survival rates as that of the eider duck also have been observed in cerebellar slices from hooded seals following both hypoxia and hypoxia combined with chemical anoxia (Ludvigsen and Folkow, unpublished observations).

Hypoxia tolerance in birds vs. mammals

Previous studies suggest that birds in general are more hypoxia-tolerant than mammals (for review see Faraci (1991)). The higher tolerance of birds to (e.g. high altitude) hypoxia in relation to brain function has been explained in terms of several factors, one of which is a more efficient vascular delivery of oxygen to their brain. Table 1 of paper III reveals that cerebellar neurons of both duck and chicken displayed significantly higher survival rates in

hypoxia and chemical anoxia than did rat cerebellar neurons under similar *in vitro* conditions. Thus, these data suggest that enhanced intrinsic neuronal hypoxia tolerance may be added to the list of adaptations that make birds in general more hypoxia-tolerant than mammals.

Mechanisms involved in neuronal hypoxia protection

Thus far the major conclusions are that neurons of the diving species, the hooded seal and the eider duck, display enhanced hypoxia tolerance compared to neurons from non-diving control animals, which is due to intrinsic properties of the neurons themselves. With this notion in mind, the obvious question is: what are the mechanisms behind the neuronal hypoxia tolerance of these animals?

Anaerobic capacity in hooded seal neurons

Cerebral glycogen stores in the Weddell seal are 2-3 times higher than those of non-diving mammals (Kerem et al., 1973), which in effect may render the tissue less dependent on blood flow rates during asphyxic hypoxia and lend enhanced anaerobic capacity. It has also been shown that the brain of the Weddell seal has higher LDH activity than the ox (Murphy et al., 1980). High LDH activity is indicative of improved anaerobic capacity since enzyme levels correlate with outstanding hypoxia tolerance in invertebrates (Hochachka, 1980).

The elevated hypoxia tolerance of hooded seal cortical tissue (paper I) presumably allowed the use of unusually thick (680 μm) brain slices (paper II). These spontaneously active slices were most likely anoxic in their core as the O_2-tension was rapidly decreasing from the outer surface down to a depth of 200 μm where it was close to the lower detection level of the measuring device, even during normoxia. This implies that a considerable number of the neurons in these slices must have been metabolizing anaerobically at any time. Furthermore, some of the slices were able to maintain activity for a considerable time in severe hypoxia, during which an even greater number of neurons presumably were anoxic. In addition, cerebellar slices from hooded seals that must have been metabolizing anaerobically in hypoxia due to the presence of NaCN, showed a high survival rate, some slices even

maintaining a significant level of activity during this exposure (Ludvigsen and Folkow, unpublished observations). Thus hooded seal neurons seem to have a capacity for anaerobic metabolism that is adequate to keep the neurons viable, and some neurons even active, for some time during severe lack of oxygen.

Anaerobic capacity in avian neurons

Bryan and Jones (1980) studied cerebral energy metabolism in intact diving and non-diving birds during hypoxia and apnoeic asphyxia to investigate if the hypoxia tolerance of the diving birds may be explained in terms of biochemical properties. To do this they recorded changes in cerebral mitochondrial NADH levels as an indication of the oxidation-reduction state of mitochondrial respiratory chain carriers (figure 3). Based on these recordings they concluded that differences in hypoxia tolerance are not likely due to biochemical adjustments, neither an ability to continue oxidative phosphorylation at levels of hypoxia not tolerated by non-divers, nor enhanced anaerobic ATP production. However, this conclusion was only based on the state of the mitochondria, and the possibility of cytosolic pathways being able to account for differences in anaerobic capacity were not discussed (Hochachka and Murphy, 1979). Furthermore, a similar study in ducks by Bickler and coworkers (Bickler et al., 1989) concluded that hypoxia tolerance due to anaerobiosis and/or hypometabolism *is* a likely adaptation in hypoxia tolerant birds, and Blix and From (1971) found evidence that the eider duck possess cerebral biochemical adaptations of anaerobic nature, in that brain tissue of these diving birds only contains one of the lactate dehydrogenase isoenzymes, LDH_5. LDH_5 favours formation of lactate from pyruvate (Bittar et al., 1996) and is in mammals typically found in glycolytic tissue such as skeletal muscle (Blix and From, 1971). In paper III, the level of activity in cerebellar slices that were able to maintain activity in severe hypoxia was significantly higher in duck than in chicken slices (the situation was similar during recovery

from hypoxia). As with hooded seal cortical slices, a large part of the neurons in the cerebellar slices from both species were presumably anoxic in hypoxia, and as such the level of activity is indicative of higher anaerobic capacity in duck slices than in chicken slices. Furthermore, rate of survival as well as the level of ∫SCA in slices recovering from chemical anoxia was higher for ducks than for chicken, and this survival must have been fuelled by anaerobic ATP-production since presence of NaCN would inhibit oxidative phosphorylation. As such, this provides support to the possibility that biochemical adjustments in the form of elevated anaerobic capacity are partly responsible for the higher neuronal hypoxia tolerance of birds.

Neuronal reconfiguration?

Spontaneously active duck cerebellar slices exposed to hypoxia or chemical anoxia could be divided into two categories according to their response to the insult. In the majority of slices the spontaneous activity disappeared more or less completely within 2-8 min of introduction of hypoxia/anoxia, while in the few remaining slices neuronal activity was maintained at various levels throughout the entire hypoxic exposure. A large part of the eider duck slices showing the former response survived, i.e. regained activity upon reoxygenation, while chicken and rat slices survived in lower proportions and with much weaker activity levels in recovery. The rapid loss of activity with subsequent recovery after end of hypoxia/anoxia may reflect a hypoxia-triggered protective shut-down of neural activity, similar to that described for anoxia-tolerant vertebrates such as turtles (Doll et al., 1991; Bickler and Donohoe, 2002; Lutz et al., 2003; Bickler, 2004; Bickler and Buck, 2007). However, a diving duck cannot enter a state of near coma as turtles do during periods of anoxia. Rather, diving ducks must remain active and coordinated in order to avoid drowning, which is only possible if a minimum of neural circuits are able to remain active despite severe hypoxia.

A minimum of control of homeostatic mechanisms must be maintained, even for a dormant turtle, and partial and selective neuronal 'shut-down' has been proposed to be important in the turtle brain (Bickler and Donohoe, 2002) as well as in diving mammals (Ramirez et al., 2007). This proposal is supported by the fact that electroencephalogram recordings from anoxic turtles reveal substantial variation over periods of several hours (Fernandes et al., 1997) (figure 4), and the hypoxic response of individual mammalian neurons is very diverse even within restricted neuronal populations. For instance, in the mammalian respiratory network, a functional reconfiguration is achieved by balance between neurons displaying differential hypoxic responses (hyperpolarizing vs. bursting) in such a way that functional integrity is maintained (Pena et al., 2004; Ramirez et al., 2007). These diverse neuronal responses may be the result of mechanisms similar to those contributing to neuronal hypoxic survival in hypoxia-tolerant vertebrates (for reviews see Lutz et al., 2003; Bickler, 2004; Bickler and Buck, 2007). Similarly, the dual hypoxic response observed in avian cerebellar neurons may reflect reconfiguration mechanisms.

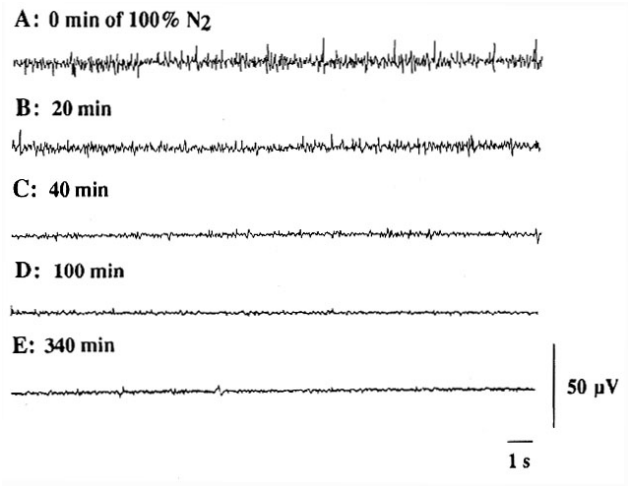

Figure 4: Effects of anoxia on EEG recordings in turtle. **A:** 0 min anoxia, representing normoxic EEG. **B-E:** 20, 40, 100 and 340 min anoxia. Bursts of activity that interrupt the depressed activity state is present at >100 min anoxia. From Fernandes et al. (1997), reproduced with permission from APS.

Neuroprotective K^+ channels?

As already outlined, a dual response to hypoxia was observed in duck cerebellar slices (paper III). The most common neuronal behaviour, in which activity subsided during hypoxia and returned upon recovery with normoxia, suggests that these neurons employ a defense-mechanism in which they are inactivated during hypoxia. In a variety of central mammalian hypoxia-sensitive neurons, a K^+ channel-mediated hyperpolarization precedes the terminal anoxic depolarization (Misgeld and Frotscher, 1982; Hansen, 1985; Haddad and Jiang, 1993). This hyperpolarization reduces neuronal activity and ion fluxes across the membrane, reducing the activity of ion pumps that consume about 50% of the energy supplied to the brain (Hansen, 1985; Hochachka, 1986). Accordingly, if this anoxic hyperpolarization can persist for some time it may have a protective effect. The cellular mechanism leading to activation of the anoxic K^+ conductance seems to be ATP-sensisitve K^+ (K_{ATP}) channels (Mourre et al., 1989; Luhmann and Heinemann, 1992) (for further references, see Kulik et al., 2002). Results from pharmacological manipulation of K_{ATP} channels indicate that these channels offer partial cellular protection against energy and/or oxygen deprivation in both cardiomyocytes (see e.g. Kane et al., 2005) and neurons (Ben-Ari et al., 1990; Abele and Miller, 1990; Wind et al., 1997; Ballanyi and Kulik, 1998; Pek-Scott and Lutz, 1998; Garcia de et al., 1999; Ballanyi, 2004) (figure 5).

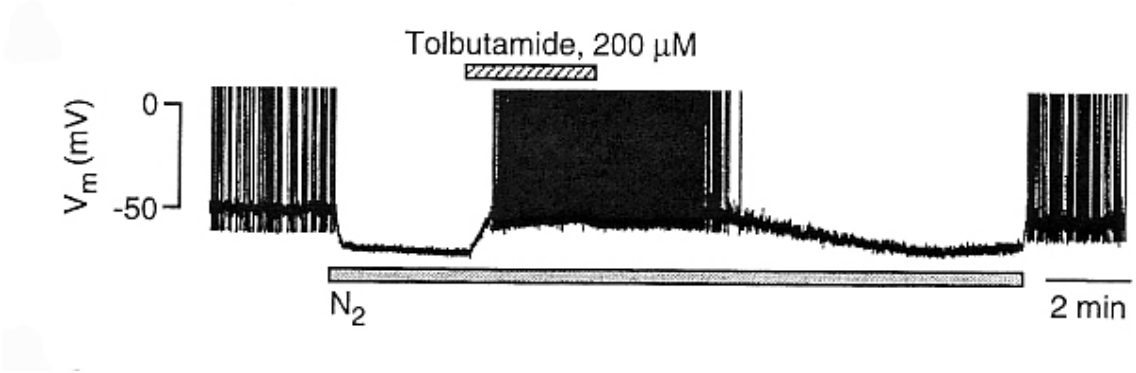

Figure 5: Intracellular recording of a spontaneously active dorsal vagal neuron from rat. Anoxia results in a sustained hyperpolarization and concomitant suppression of action potential discharge. Adding the K_{ATP} channel blocker tolbutamide reverses suppression of activity. From (Ballanyi and Kulik, 1998), reproduced with permission from John Wiley & Sons.

In eider duck cerebellar slices, the K_{ATP} channel opener diazoxide had a depressing effect on the activity of normoxic, spontaneously active neurons, suggesting the presence of such channels (paper IV). Thus, adding the drug resulted in a gradual and significant drop in activity. However, the K_{ATP} channel blocker tolbutamide had only a weak activating effect on spontaneously active neurons after they had displayed loss of activity in response to hypoxia/chemical anoxia. The tolbutamide-treated slices also tended to display a lower level of activity in the normoxic recovery period compared to control slices, but this difference was only significant at 30 min of recovery. These results suggest that K_{ATP} channels to some extent influence the excitability of silenced cerebellar neurons in the eider duck, but they seem to have a rather limited role in the neuronal shut down that takes place in most slices in hypoxia. It is likely that the observed response is due to a hyperpolarisation of the neurons that make them insensitive for activating signals, much in the same manner as Purkinje neurons in mice show typical K_{ATP}-mediated hyperpolarization and increase in membrane K^+ conductance in response to hypoxia, chemical anoxia and diazoxide (Ballanyi, 2004). It is also worth mentioning that preliminary results from identical experiments with hooded seal

slices also revealed a weak response to tolbutamide, but further experiments are needed to validate this.

Jiang et al. (1992) (figure 6) found that K_{ATP} channels are poorly expressed in the anoxia-tolerant turtle and hypoxia-tolerant newborn rat in comparison to the more hypoxia-sensitive adult rat. It is tempting to speculate if this is a trait in all hypoxia/anoxia tolerant animals. This presents us with an intriguing question; do neurons in hypoxia-sensitive animals rely on K_{ATP} channels to survive brief periods of hypoxia while neurons in hypoxia-/anoxia-tolerant animals use a different strategy to endure longer periods? If so, it could explain the weak response to tolbutamide in the eider duck cerebellar slices.

In contrast to the study by Jiang and colleagues (Jiang et al., 1992), a review by Bickler and Buck (Bickler and Buck, 2007) discusses the possibility that K_{ATP} channels *do* play a role in the anoxia tolerance of turtles. The authors suggest that reduction of NMDA receptor activity in the anoxic turtle brain may involve mitochondrial K_{ATP} (mK_{ATP}) channels in a similar fashion as in the heart. Activation of mK_{ATP} channels in the heart results in influx of K^+ into the mitochondria, which in turn results in elevated intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Holmuhamedov et al., 1998). In anoxic turtle neurons, NMDA-receptors are inactivated in a dose-dependent manner with increasing $[Ca^{2+}]_i$ (Bickler et al., 2000), and studies have shown that activation of mK_{ATP} channels reduce whole-cell NMDA receptor currents by about 40% (Bickler and Buck, 2007). The reduction of NMDA receptor currents is abolished by K_{ATP} blockers, both general and specific. Furthermore, inactivation of Ca^{2+} by chelation reverses reductions in NMDA receptor currents, implying that $[Ca^{2+}]_i$ is necessary for mK_{ATP}-reduction of anoxic NMDA receptor activity (Bickler and Buck, 2007).

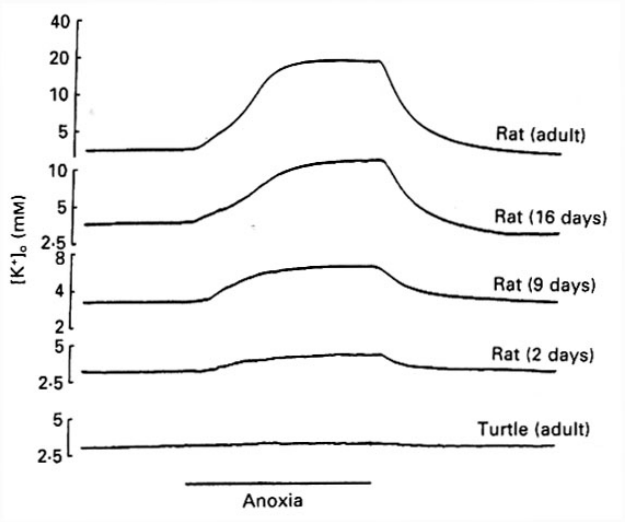

Figure 6: Anoxia-induced increase in extracellular K^+ in rats (adults and neonates) and turtles. The anoxia tolerance decreases with age in the rat (as more K^+ is released in older rats), while there is no change in extracellular K^+ in the adult turtle. Anoxia was maintained for 4 minutes in rat and 6 minutes in turtle. From (Jiang et al., 1992), reproduced with permission from Wiley-Blackwell.

Possible role of neuroglobin

A possible mechanism contributing to the elevated hypoxia tolerance of hooded seal neurons is facilitated O_2-transport to the cells by the reversibly O_2-binding protein neuroglobin (Ngb) (Burmester et al., 2000). However, investigations on Ngb levels in the hooded seal brain revealed no significant differences compared to those of rats and mice (Mitz et al., 2009). But while Ngb is restricted almost exclusively to neurons in non-diving mammals (Reuss et al., 2002; Wystub et al., 2003; Hankeln et al., 2004), it was discovered that in the hooded seal brain the protein, as well as cytochrome oxidase c (Cyt c) is mainly located in astrocytes (Mitz et al., 2009). As both Ngb and Cyt c are markers of oxidative metabolism (see Mitz et al., 2009), this unusual observation led the authors to hypothesize that cerebral metabolism in seals is fundamentally different from that of terrestrial mammals, in that seal astrocytes appear to be highly oxidative while seal neurons largely may function anaerobically. This could in part explain the high hypoxia tolerance of hooded seal neurons demonstrated in paper I.

Expression of inhibitory and excitatory neurotransmitters

Since breakdown of the inhibitory neurotransmitter GABA is O_2 dependent (Nilsson, 1992), it is plausible to expect that hypoxia triggers a general increase in brain levels of GABA. This has been confirmed in e.g. anoxia tolerant ectotherms, where elevated levels of GABA inhibit neuronal activity and reduces energy consumption, thereby prolonging survival in hypoxia (Lutz and Nilsson, 2004) (figure 7). Also, a 27% increase in the brain level of GABA has been found to occur in tilapia (*Oreochromis mossambicus*) following two hours of anoxia at 20°C (van Ginneken et al., 1996), but it is unknown whether this increase was a response to diminished O_2 levels. Accumulation of GABA has also been observed in anoxia-intolerant species like brown anole lizards (*Anolis sagrei*) (Nilsson et al., 1991) and dogs (Tews et al., 1963), but to a lesser extent.

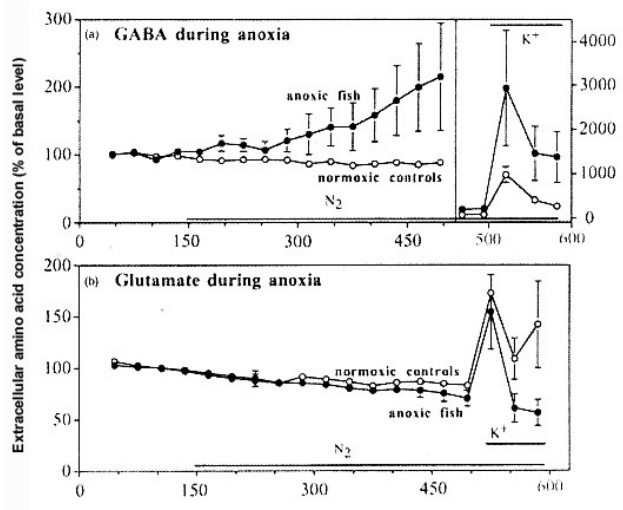

Figure 7: Microdialysis measurements of extracellular neurotransmitters in crucian carp telencephalon showing the effect of anoxia (and high K^+ concentration). There is a large increase in levels of the inhibitory neurotransmitter GABA (upper graph) and a small decrease in levels of the excitatory neurotransmitter glutamate (lower graph) in response to anoxia. From (Lutz and Nilsson, 2004), reproduced with permission from John Wiley & Sons.

When investigating correlations between GABA levels and degree of neuronal metabolic depression in the epaulette shark, Mulvey and Renshaw (2009) found no general increase in levels of GABA following hypoxic preconditioning. Rather, they found that levels

of GABA changed heterogeneously, and this was not to be expected if there is a general lack of O_2-dependent breakdown of GABA. Based on this the authors suggested that regional distribution changes provide neuroprotective mechanisms that ensure that vital regions are protected from neuronal damage during hypoxia and reoxygenation. This is a mechanism similar to the hypothesized principle of neuronal network reconfiguration by Ramirez and colleagues (Ramirez et al., 2007).

In addition to an increase in the level of the inhibitory neurotransmitter GABA, anoxia-tolerant ectotherms show a decrease in the level of excitatory neurotransmitters such as glutamate and dopamine during anoxia (Nilsson and Lutz, 1991; Milton and Lutz, 1998; Milton et al., 2002) (figure 7). The effect of this is the same as that of increasing the level of GABA, namely depressing neuronal activity and conserving energy. Both release and reuptake of the neurotransmitters is maintained at a reduced level in accordance with the lowered energy expenditure (Milton and Lutz, 1998; Milton et al., 2002), securing functional integrity of the neuronal network.

Reduced ion channel density and/or activity

Reducing ion permeability will significantly reduce the energy consumption of neurons, as ion pumping accounts for more than 50% of the energy demand of a normoxic neuron (Lutz et al., 2003). Turtles seem to utilize this strategy (Lutz and Nilsson, 2004), while in the less anoxia-tolerant crucian carp investigations have revealed no reduction in ion permeability (Johansson and Nilsson, 1995; Nilsson, 2001).

Voltage-gated K^+ channels (Kv channels) are involved in determining the electrical activity of neurons, and investigations have shown that O_2 supply both down- and up-regulates gene transcription of Kv channels in the turtle brain (Prentice et al., 2003). If one

assumes that transcription and translation correlates, a reduced K^+ permeability in the anoxic turtle brain may be related to the down-regulation of ion channel gene expression.

In most neurons voltage-gated Na^+ channels are responsible for the generation of action potentials. A 42% decrease in the density of these channels has been detected in the turtle brain during anoxia (Perezpinzon et al., 1992), which probably elevates the action potential threshold causing what has been termed 'spike arrest' (Sick et al., 1993).

Activity of the Ca^{2+}-permeable NMDA receptor is reduced in the turtle brain during anoxia, activity falling by 50-60% within minutes (Bickler et al., 2000). During prolonged anoxia there is further decrease as a result of removal/internalization of receptors from the cell membrane (Bickler et al., 2000).

Hb affinity for O_2

Crucian carp and goldfish Hb has an extremely high affinity for O_2 (Lutz and Nilsson, 2004), resulting in 50% saturation at a Pa_{O2} of only 2.5 mmHg (Burggren, 1982). This allows the crucian carp to maintain normal O_2 consumption rates even when water O_2 levels drop below 10% saturation (Sollid et al., 2003). Similarly, in high altitude bird species, mutated Hb with high O_2 affinity is an important mechanism contributing to their hypoxia tolerance (Gou et al., 2007). Data on the Hb O_2-affinity of diving mammals however, show only a slightly higher affinity when compared with terrestrial mammals (Lenfant et al., 1969; Lenfant et al., 1970), which makes sense considering that these animals always breathe air at normal atmospheric pressure (Folkow and Blix, 2010).

Capillary density

The only way O_2 can be supplied from capillaries to nearby neurons is through diffusion (Nicholson, 2001), and the driving force for this diffusion is the difference in P_{O2} between

neurons and the surrounding capillaries (Xu and LaManna, 2006). This means that if an animal becomes hypoxemic, this driving force is reduced, thereby slowing down delivery of O_2. A way to compensate for this loss of driving force is to shorten diffusion distance by increasing capillary density. In fact, exposure to chronic hypoxia triggers angiogenesis in the brain of both rats (LaManna et al., 1992; Harik et al., 1996; Patt et al., 1997; Pichiule and LaManna, 2002) (figure 8) and humans (Vagnucci, Jr. and Li, 2003). As diving seals experience hypoxemia at the end of a long dive (Scholander, 1940; Qvist et al., 1986; Meir et al. 2009), having a high capillary density would be beneficial since this would ease delivery of O_2 to the brain when the animal encounters this challenge. A high capillary density has been demonstrated in the brain of the northern elephant seal (Kerem and Elsner, 1973) and in dolphins (Glezer et al., 1987) compared to non-diving mammals. Analysis of capillary density in the brain of hooded seals (paper V) revealed that this species has a capillary density similar to that of much smaller non-diving mammals. This is not to be expected from capillary density scaling laws (Dawson, 2003), and may suggest that the hooded seal benefits from a high capillary density relative to non-diving species of similar size.

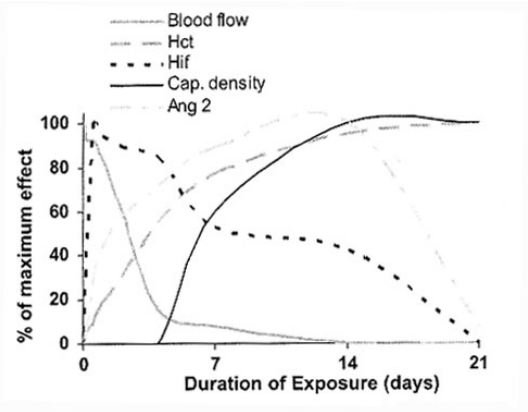

Figure 8: Relative time courses of cerebral blood flow, hematocrit (Hct), HIF-1α (Hif), capillary density and angiopoetin-2 (Ang 2) in rodents in response to chronic mild hypoxic exposure. From (Xu and LaManna, 2006), reproduced with permission from APS

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Methodological considerations

The brain slice technique and patch clamping as a method

A big step forward for those studying the brain and neuronal mechanisms came with the development of the brain slice technique. This technique was pioneered by the British scientist Henry McIlwain in the 1950s for the purpose of biochemical studies of brain tissue (Collingridge, 1995). To validate his preparations, McIlwain modified electrophysiological techniques so that they could be applied to slices of brain tissue, and found that the slices were viable and contained neurons with healthy resting membrane potentials. In 1966 a description of synaptically viable slice preparations was published for the first time (Yamamoto and Mcilwain, 1966), laying the foundation for the development of techniques such as patch clamping. The patch clamp method has expanded into many fields of biology and basic research in medicine. The technique is especially useful in the study of excitable cells such as neurons, cardiomyocytes, muscle fibers and pancreatic ß-cells. Electrophysiological studies on neurons by use of brain slices have formed the basis for much of our current understanding of neuronal mechanisms, such as those involved in learning and long-term potentiation, work that was pioneered by Per Andersen and Terje Lømo at the University of Oslo (see Lømo (2003) for a review of their discoveries).

The visual cortex as a model

The visual system consists of several processing areas that are anatomically distinct. In mammals, the area processing visual information is found in the visual cortex (VC) which is situated in the occipital lobe posterior in the cerebrum. It responds to stimulus such as color, spatial frequency, eye of origin, motion and visual disparity between the two eyes (Ng et al., 2007). In cats it has been demonstrated that neuronal activity in the VC is reduced within 1-2

minutes in hypoxia/anoxia (Kayama, 1974; Eysel, 1978). Furthermore, cortical layer V is among the most vulnerable areas to damage from hypoxia in the brain (Brierley and Graham, 1984), which was the main reason for targeting pyramidal cells of layer V of the VC for intracellular recordings in this work.

The cerebellum as a model

Several behaviours require the cerebellum for optimal performance. These include target-reaching motions, movements requiring precision timing, balance and posture control, certain forms of motor learning and reflex adaptation, and a number of tasks that involve the planning and execution of specific action sequences (Dow and Moruzzi, 1958; Ito, 1984; Ivry, 1997; Schmahmann, 1997). In terrestrial mammals cerebellar Purkinje cells are vulnerable to hypoxia and ischemia (Cervos-Navarro and Diemer, 1991; Lutz et al., 2003; Pae et al., 2005; Larsen et al., 2005), and loosing proper function of the cerebellum and the behaviours it is required for would spell disaster for any animal. This makes the cerebellum a relevant model for studying neuronal defence mechanisms against hypoxia in animals that we know experience hypoxemia.

Extracellular recordings vs. intracellular recordings

Most of the work in this project is based on extracellular recordings. This approach was in several cases preferred above intracellular recordings, for the following reasons: The present work focused on neuronal responses to hypoxia in diving versus non-diving endotherms, in which the hooded seal and eider duck were chosen as experimental models of divers. This was a natural choice because both represent specialized divers, and because the department has years of experience in studying and keeping these species in captivity. However, working with such exotic animals (exotic compared to the ordinary laboratory rats and mice) has clear

limitations. First the number of animals available is obviously limited. Both species reproduce only once a year and are difficult to capture in the field, so access to new animals was restricted to once a year. Second, the number of animals in keeping is always limited, particularly considering that they had to be maintained until reaching sub-adult age. Third, whilst intracellular recordings from mice neurons were relatively easy to perform and an easy access to new animals allowed a higher failure rate, recordings from both duck and seal neurons proved more difficult. This was further limited by the fact that we used the blind patch approach, i.e. we did not have a microscope for visual guiding of electrode placement at a cellular level. Therefore, a decision was made as to how to get the most out of the animals available, and it was decided to sacrifice resolution for a larger 'n'. Knowing that cerebellar slices would most likely display spontaneous activity well suited for extracellular recordings that are easy to perform, it was decided to use this approach in some of the studies.

General conclusions

In this work it has been demonstrated, for the first time, that both the deep diving hooded seal and the diving eider duck display enhanced neuronal hypoxia tolerance compared to non-divers, which is likely to be due to mechanisms intrinsic to the neurons themselves. It is also concluded that birds in general seem to display elevated brain hypoxia tolerance compared to non-diving mammals. Mechanisms responsible for intrinsic neuronal hypoxia-tolerance include (1) enhanced anaerobic capacity as judged from neuronal survival following chemical anoxia in both species, and (2) K_{ATP}-induced shut down of neuronal activity, although these channels appears to only partially contribute to hypoxia tolerance, as response to the channel blocker tolbutamide was not evident in neither eider duck nor preliminary hooded seal experiments. An additional possible mechanism has been suggested in a related study, where it was found that the respiratory protein Ngb together with mitochondria in the brain of hooded seals are located mainly in astrocytes and not in the neurons themselves. This is the opposite of the situation in e.g. rats and mice, and it is suggested that this may contribute to the hypoxia tolerance of hooded seal neurons as it implies that neurons metabolize largely anaerobically (Mitz et al., 2009). The brain of hooded seal was found to have a capillary density similar to that reported in the literature for smaller non-diving species. This was not to be expected according to capillary density scaling laws, and implies that the hooded seal benefits from a higher capillary density than that of non-diving species. The hypoxia tolerance of hooded seal neurons presumably allowed the use of unusually thick slices in which extracellular recordings revealed the presence of spontaneous, oscillating network activity resembling activity in the awake brain. The implications of this are that, contrary to earlier beliefs, the mammalian cerebral cortex is capable of initiating network oscillations independent of input from subcortical structures. Eider duck cerebellar slices displayed a dual

response to hypoxia, and identical (so far unpublished) observations were made in hooded seal experiments. It may be that this response reflects a network reconfiguration during hypoxia in which most neurons shut down activity while some stay active, possibly in order to maintain necessary brain function.

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