## Paper I

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# THE ROLE OF GLYCOGEN, GLUCOSE AND LACTATE IN NEURONAL ACTIVITY DURING HYPOXIA IN THE HOODED SEAL (*CYSTOPHORA CRISTATA*) BRAIN

**N. U. CZECH-DAMAL,[a] S. J. GEISELER,[b] M. L. M. HOFF,[a] R. SCHLIEP,[a] J.-M. RAMIREZ,[c] L. P. FOLKOW[b] AND T. BURMESTER[a]***

[a] *Institute of Zoology and Zoological Museum, Biocenter Grindel, University of Hamburg, Martin-Luther-King-Platz 3, D-20146 Hamburg, Germany*

[b] *Department of Arctic and Marine Biology, University of Tromsø – The Arctic University of Norway, NO-9037 Tromsø, Norway*

[c] *Department of Neurological Surgery, Seattle Children's Research Institute Center for Integrative Brain Research, University of Washington, Seattle, WA, USA*

**Abstract—The brains of diving mammals are repeatedly exposed to hypoxic conditions during diving. Brain neurons of the hooded seal (*Cystophora cristata*) have been shown to be more hypoxia tolerant than those of mice, but the underlying mechanisms are not clear. Here we investigated the roles of different metabolic substrates for maintenance of neuronal activity and integrity, by comparing the *in vitro* spontaneous neuronal activity of brain slices from layer V of the visual cortex of hooded seals with those in mice (*Mus musculus*). Studies were conducted by manipulating the composition of the artificial cerebrospinal fluid (aCSF), containing either 10 mM glucose, or 20 mM lactate, or no external carbohydrate supply (aglycemia). Normoxic, hypoxic and ischemic conditions were applied. The lack of glucose or the application of lactate in the aCSF containing no glucose had little effect on the neuronal activity of seal neurons in either normoxia or hypoxia, while neurons from mice survived in hypoxia only few minutes regardless of the composition of the aCSF. We propose that seal neurons have higher intrinsic energy stores. Indeed, we found about three times higher glycogen stores in the seal brain (∼4.1 ng per µg total protein in the seal cerebrum) than in the mouse brain. Notably, in aCSF containing no glucose, seal neurons can tolerate 20 mM lactate while in mouse neuronal activity vanished after few minutes even in normoxia. This can be considered as an adaptation to long dives, during which lactate accumulates in the blood.** **© 2014 IBRO. Published by Elsevier Ltd. All rights reserved.**

**Key words: astrocyte–neuron lactate shuttle, brain, glycogen, lactate, hypoxia, ischemia.**

## INTRODUCTION

Hooded seals (*Cystophora cristata*) display an impressive diving capacity in coping with regular exposure to hypoxia during dives lasting for up to one hour (Folkow and Blix, 1999). It is widely accepted that various combinations of behavioral, anatomical and physiological adaptations contribute to the remarkable dive capacity and hypoxia-tolerance of many aquatic mammals (Blix and Folkow, 1983; Butler and Jones, 1997; Butler, 2004; Ramirez et al., 2007; Ponganis, 2011; Davis, 2014; Larson et al., 2014). These adaptations include enhanced $O_2$ stores, reflected by high levels of the respiratory proteins hemoglobin and myoglobin and a large blood volume, as well as enhanced capacity for anaerobic metabolism combined with cardiovascular adjustments involving bradycardia and peripheral vasoconstriction (Scholander, 1940; Zapol et al., 1979; Blix et al., 1983; Folkow and Blix, 2010; Ponganis, 2011).

Reduced oxygen-supply (hypoxia) usually has a detrimental impact on the mammalian brain. By contrast, brains of diving mammals and birds may survive extended periods of systemic hypoxia without obvious damage (Butler and Jones, 1997; Butler, 2004; Ramirez et al., 2007; Larson et al., 2014). This is partly due to the redistribution of blood flow, which is maintained to vulnerable organs (heart and brain) at the expense of most other tissues (Ramirez et al., 2007; Folkow and Blix, 2010). In addition, electrophysiological studies demonstrated that under *in vitro* conditions neurons from the brain of the hooded seal remained 4–6 times longer active in severe hypoxia compared to mice neurons, and partly persisted for up to 1 h (Folkow et al., 2008; Ramirez et al., 2011). This raises the question about the mechanisms and defense strategies that enable the seal's brain to resist the impact of hypoxia.

Brain function depends on an adequate supply of energy substrates. The main substrate that fuels the mammalian brain is glucose. However, the views on the mechanisms that work on the cellular level differ. According to the astrocyte–neuron lactate shuttle (ANLS) hypothesis, in the brain of man (and other terrestrial mammals), glycolysis dominates in astrocytes, whereas neurons work largely aerobically and are fueled mainly by lactate from the astrocytes, which is preferred

*Corresponding author. Tel: +49-40-42838-3913; fax: +49-40-42838-3937.
E-mail address: thorsten.burmester@uni-hamburg.de (T. Burmester).

*Abbreviations:* aCSF, artificial cerebrospinal fluid; ANLS, astrocyte–neuron lactate shuttle; Ngb, neuroglobin; Pygb, brain glycogen phosphorylase; SNA, spontaneous neuronal activity.

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over glucose (Magistretti et al., 1994; Itoh et al., 2003; Pellerin, 2005). The ANLS hypothesis has stimulated a still ongoing debate on the relative roles of glucose and lactate in the brain (Pellerin, 2005, 2010). The ANLS hypothesis is supported, among others, by electrophysiological studies showing that under conditions of hypoxia and reoxygenation, rodent neurons possess a higher neuronal recovery rate in artificial cerebrospinal fluid (aCSF) with lactate than with glucose (Schurr et al., 1997a,b, 1999; Schurr, 2006). In this context, the view on lactate in the brain has changed: While for a long time lactate has been considered as signature of hypoxic brain damage, e.g. after ischemia, its function as an alternative energy source under aerobic conditions has come into focus (Schurr, 2006; Dienel, 2012).

Mitz et al. (2009) suggested that the seal brain employs an alternative strategy to ANLS to better survive hypoxic periods. Compared to the brains of terrestrial mammals, in the brain of the hooded seal the mitochondrial protein cytochrome C and the respiratory protein neuroglobin (Ngb) are shifted from neurons to astrocytes. A similar observation has recently been made in the brain of the harp seal (*Pagophilus groenlandicus*) (Schneuer et al., 2012). This finding led to the assumption of a ''reverse'' ANLS in the seal's brain, suggesting that anaerobic glycolysis predominantly occurs in seal neurons and that the produced lactate is taken up and metabolized aerobically by astrocytes. Neuronal activity based primarily on anaerobic metabolism would, on the one hand, reduce oxygen dependency and, on the other hand, may also enhance protection from oxidative stress, which occurs from mitochondrial activity during reoxygenation of the brain after surfacing (Mitz et al., 2009). Notably, brains of cetaceans do not show a redistribution of cytochrome c and Ngb, as it was observed in the seals, and instead possess much higher levels of Ngb, which probably supports the oxidative metabolism (Schneuer et al., 2012).

A reverse ANLS shuttle would require various metabolic changes. For example, the neurons of the seal brain would be expected to be better adapted to anaerobic glycolysis than the neurons of terrestrial mammals and could display differences in lactate utilization. Here we have compared spontaneous neuronal activity (SNA) of brain slices of hooded seals and mice in aCSF supplemented with either glucose or lactate under normoxia or hypoxia, as well as under ischemic conditions. To evaluate the relative role of stored glycogen, we further analyzed the glycogen content and the mRNA levels of brain glycogen phosphorylase (Pygb), an enzyme catalyzing the rate-determining step in glycogen degradation, in the neocortex and the cerebellum.

## EXPERIMENTAL PROCEDURES

### Animals and sample preparation

Hooded seals (*C. cristata*) were live-captured from large breeding colonies in the pack ice of the Greenland Sea, in conjunction with expeditions with the Norwegian research vessel ''Jan Mayen'' in March/April between the years 2007 and 2010 under permits issued by Norwegian and Greenland authorities (Norway: 06/21058, 08/531, 09/4764, 09/23225; Greenland: JTF.j.nr. 55.Dan.9-7 and JTF.j.nr.Grønland.9). Animals were kept in approved facilities of the Department of Arctic and Marine Biology, University of Tromsø – The Arctic University of Norway. Brain samples were obtained from juvenile hooded seals ($n = 7$; 1.5–2.5 years) immediately after euthanasia (bleeding and decapitation in deep anesthesia (intramuscular/intravenous injection of zolazepam/tiletamine, 2.0–3.0 mg per kg of body mass [Zoletil Forte Vet., Virbac S.A., France])) that was conducted for a range of scientific purposes, including those of the present study. The procedure was approved by the authorities at the University of Tromsø (permit numbers: AAB/06, 18/09, 13/10). Mice were kept in approved facilities of the Department of Neurophysiology, University of Hamburg, Germany. Brain samples were obtained from adult NMRI mice ($n = 41$, P21–P35) of both sexes. Isoflurane (Forene, Abbott, Germany) was used for inhalational anesthesia and the animals were subsequently decapitated in accordance with the European guidelines for the care and use of animals in scientific experiments. After decapitation the brains of seals and mice were removed, placed in cooled (4 °C) glucose-aCSF saturated with 95%$O_2$–5%$CO_2$ and further processed as described below.

### aCSF

All aCSF solutions contained 128 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM $NaHCO_3$ and 0.5 mM $NaH_2PO_4$. Glucose-aCSF contained additional 10 mM D-glucose and 20 mM sucrose. Lactate-aCSF included 20 mM L-lactate (as an equicaloric replacement for glucose) and 10 mM sucrose, and glucose-free aCSF contained 30 mM sucrose. All solutions were adjusted to pH 7.4.

### Slice preparation and extracellular recordings

Neocortical brain samples of the visual cortex were glued with a supporting agar block to the stage of a Leica vibroslicer (VT1000s or VT1200s). 400-μm-thick slices were cut and allowed to recover in a holding chamber containing oxygenated glucose-aCSF at room temperature for at least 30 min. Before recording spontaneous extracellular activity slices were transferred to a custom-made organ bath superfused with re-circulating thermostatically controlled oxygenated glucose-aCSF at a rate of ~30 ml/min. The slices were allowed to adjust to 34 ± 0.5 °C for at least 20 min. Neuronal population activity recordings were made in the visual neocortical layer V using aCSF-filled borosilicate glass electrodes that were positioned on the surface of the slices with a Leitz (Wetzlar, Germany) or a Sutter MP225 (Sutter Instrument Co., Novato, CA, USA) manipulator. The first 10 min of recording were discarded to assure that the observed activity was not caused by injury discharge associated with the positioning of the electrode. Injury discharges typically displayed a sudden onset of high levels of action

potential discharge that disappeared within less than 5 min. The signals were amplified 1,000 times and filtered (low pass 100 Hz, high pass 3 kHz) using a Grass (P55, Grass Telefactor, West Warwick, RI, USA) and a Gould Universal (Gould Instrument Systems, Inc., Valley View, OH, USA) amplifier. Each experiment lasted for approximately 2 h. The first 20–30 min of normoxia was recorded either in glucose aCSF or 10 min in glucose and 20 min in lactate aCSF. One hour of severe hypoxia was introduced by switching the gas flow from 95%$O_2$–5%$CO_2$ to 95%$N_2$–5%$CO_2$ and reoxygenation by switching back to 95%$O_2$–5%$CO_2$ (Fig. 1B, C). Aglycemia was introduced by changing from glucose-aCSF to glucose-free aCSF while maintaining normoxia (Fig. 1D) and ischemia by changing from glucose-aCSF to glucose-free aCSF with the simultaneous onset of hypoxia (Fig. 1E). Slices that were subjected to 60 min of normoxia in the respective aCSF acted as negative controls (Fig. 1A). For each measurement, a new brain slice was employed. During recording sessions no additional $K^+$ was added, nor were the extracellular $Ca^{2+}$ or $Mg^{2+}$ concentrations decreased.

All recordings were stored on a personal computer using a PowerLab 4/25 data acquisition system and analyzed offline using Chart 5 for Windows software (ADInstruments Ltd., Chalgrove, UK). Spike numbers of SNA above a pre-determined threshold level (7 μV) were integrated over ±0.5 min time intervals. Average SNA levels are expressed in relative values, in relation to the baseline SNA, averaged over the last 10 min in normoxia prior to start of hypoxia or ischemia, which was set to 100%. Statistical analyses were performed with SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA) or GraphPad Prism 6 for Windows, Version 6.01 (La Jolla California USA). $p$ values $< 0.05$ were considered significant.

### Measurement of glycogen content

Brain samples of the cortex and cerebellum of hooded seals ($n$ = 3) and mice ($n$ = 3) were collected and washed in glucose-free aCSF. They were snap-frozen in liquid nitrogen and stored at −80 °C. Prior to use, samples were homogenized in liquid nitrogen and diluted in 50 volumes of HPLC-water. After 5 min of heat inactivation at 70 °C and centrifugation at 13,000 rpm for 3 min, samples were stored at −20 °C. Protein levels were measured using the BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). Glycogen measurement was according to the manufacturer's protocol of the Glycogen Assay Kit (Abnova, Taipei City, Taiwan). Fluorometric analyses were performed in a 96-well microplate format using a DTX 880 Multimode Detector microplate reader (Beckman Coulter, Krefeld, Germany) with a 540-nm filter. Data were normalized to total protein. The results were statistically analyzed by unpaired T-test employing SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

### Cloning, sequencing and real-time RT-PCR (qRT-PCR) of PYGB-cDNA

Brain samples of the cerebral cortex and the cerebellum (hooded seal $n$ = 3, mouse $n$ = 5) were stored in RNAlater (Qiagen, Hilden, Germany) at −80 °C. Total RNA was extracted with peqGOLD Trifast (PEQLAB, Erlangen, Germany) and purified using RNeasy® Mini Kit (Qiagen) following the manufacturer's protocol. An additional on-column digestion with RNase-Free DNase (Qiagen) was performed. Quantity and quality of the RNA were checked photometrically and by gel electrophoresis. First-strand cDNA synthesis was carried out using Fermentas RevertAid H Minus reverse transcriptase kit (Thermo Scientific, Germany) according to the manufacturer's protocol. For the amplification of

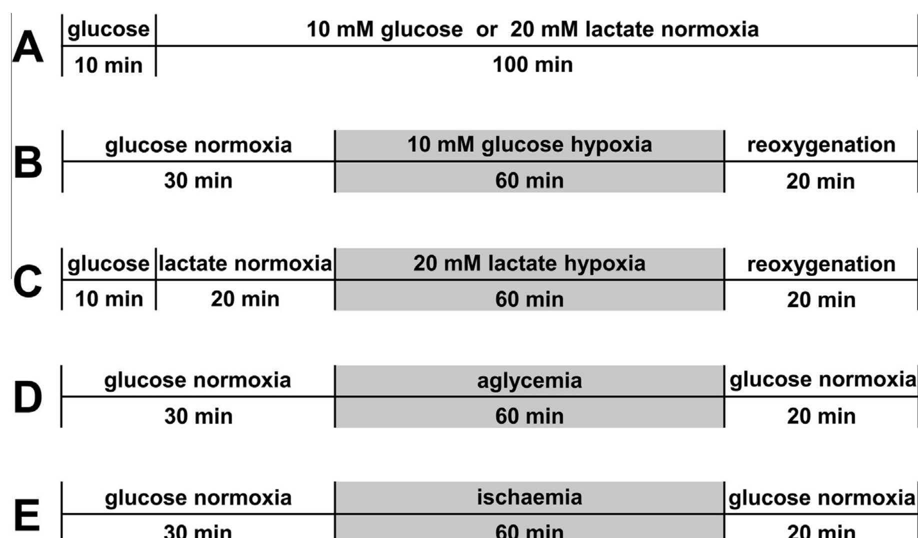


**Fig. 1.** Experimental design and time sequence of the different conditions used during extracellular recordings. (A) Control experiments in 10 mM glucose or 20 mM lactate in normoxia. (B) Hypoxic experiments with 10 mM glucose and (C) with 20 mM lactate. (D) Experiments under aglycemic and (E) ischemic conditions.

partial CDS glycogenphosphorylase (PYGB) sequences, degenerated oligonucleotide primers were designed according to the conserved regions of aligned mammalian PYGB-cDNA sequences: 5′-AGATTRTCAA TGGCTGGCA-3′ and 5′-CCATGTTRATYCKCTTRCAG TC-3′. The PCR fragments were cloned into the pGEM-T Easy/JM109 system (Promega, Mannheim, Germany) and sequenced by a commercial service (GATC, Konstanz, Germany). For qRT-PCR reverse transcription was performed with 1.5-μg total RNA in a 20-μl reaction using Fermentas RevertAid H Minus reverse transcriptase kit (Thermo Scientific, Germany). Pairs of PYGB primers flanking a 164 bp and 154 bp long CDS region were designed for hooded seal and mouse, respectively. They were: 5′-GAGAAAGTGGACT GGGACAAGGC-3′ and 5′-AGGTGCCTCTGGTTG ATGGC-3′ for hooded seal, and 5′-TGGTACTCGCTATG CCCTACGACA-3′ and 5′-AAGTTCCTATCCAGGACA GCTTCGA-3′ for mouse. qRT-PCR amplification was carried out on the ABI 7500 real-time PCR system with the Power SYBR green master mix (Applied Biosystems, Darmstadt, Germany) using a 40-cycle protocol (95 °C for 15 s, 58 °C for 15 s, 72 °C for 30 s). Fluorescence was measured at the end of each amplification cycle. PCR was performed in triplicates containing a final cDNA amount equivalent to 34.1-ng total RNA and a final primer concentration of 0.23 μM in a 20 μl solution. Additionally, negative controls without cDNA were included. Specificity of the amplification reaction was analyzed in dissociation curves and by gel electrophoresis. Absolute mRNA copies were calculated with the 7500 System Sequence Detection Software (Applied Biosystems, Darmstadt, Germany) by means of the standard curve method by dilutions ($10^8$–$10^3$) of the recombinant plasmid. Large ribosomal phosphoprotein P0 (RPLP0) mRNA levels in the samples were determined as a sample control. PYGB expression levels of each organism were normalized per total RNA quantity. The results were analyzed by unpaired *t*-test employing GraphPad Prism 6 for Windows, Version 6.01 (La Jolla California USA).

## RESULTS

### Responses of mouse and seal brain neurons to glucose and lactate at normoxia and hypoxia

Extracellular recordings were obtained from isolated neocortical slices of layer V of the primary visual neocortex (V1) from hooded seal and mouse. For better comparison with other species and in contrast to former electrophysiological studies of the hooded seal (Folkow et al., 2008; Ramirez et al., 2011) we used 400-μm-thick isolated seal brain slices instead of 680 μm, and aCSF with 10 mM glucose instead of 30 mM glucose. At normoxia, seal ($n$ = 4 slices) and mouse ($n$ = 6 slices) slices displayed consistently SNA that exceeded 7 μV throughout the exposure to 10 mM glucose in aCSF for 2 h (Fig. 2). At exposure to 20 mM lactate, the brain slices of seals ($n$ = 5 slices) displayed a sudden onset of high levels of action potentials after 15–17 min for 1–2 min, but SNA returned to baseline activity for the remainder of the control period. Thus, seal slices were kept in 20 mM lactate for at least 20 min before exposure to hypoxia. During the whole period, relative SNA levels of seal brain slices in glucose, seal brain slices in lactate and mouse brain slices in glucose were not significantly different (a two-way ANOVA with Bonferroni correction for multiple comparisons and Mann–Whitney *U*-tests). In the brain slices from mice ($n$ = 10 slices), SNA disappeared after 15-min incubation in lactate and thus no further recordings were obtained.

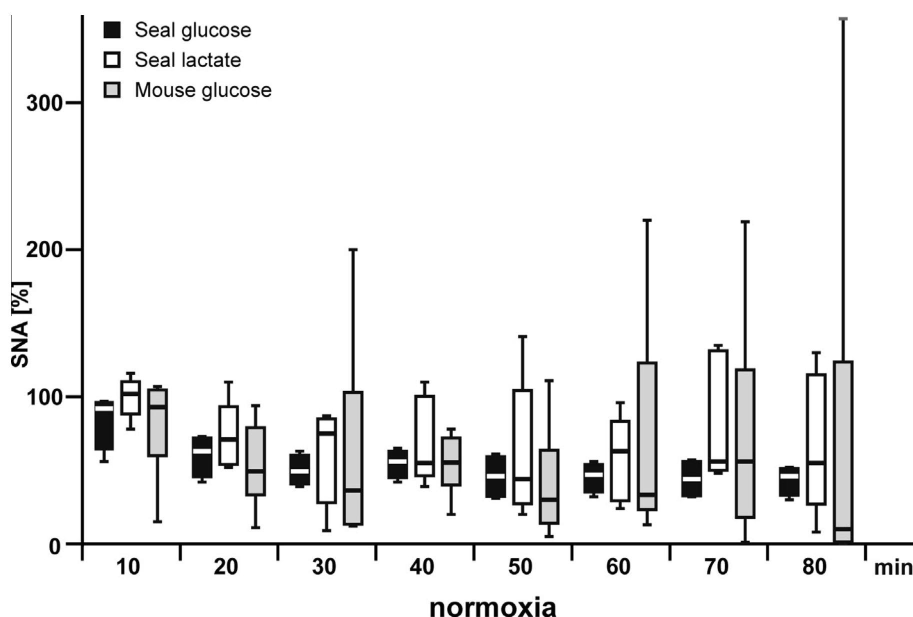


**Fig. 2.** Relative spontaneous neuronal activity (SNA) in isolated cortical slices during 80-min normoxia in 10 mM glucose (black; $n$ = 4) and 20 mM lactate (white; $n$ = 5) in hooded seal, and in 10 mM glucose in mouse (gray; $n$ = 6). SNA was averaged for 10 min prior to the start and was set to 100%. Medians, interquartile ranges, and minima and maxima are given.

In response to exposure to hypoxia for 60 min, seal slices displayed three different types of responses in glucose as well as in lactate: *i.* average SNA levels remained either unaltered compared to baseline activity or maintained some tonic spiking activity, *ii.* activity disappeared either partially or completely during hypoxia but recovered upon reoxygenation, or *iii.* activity was lost without recovery (Fig. 3). In addition, some slices exhibited at the start of hypoxia a 1–2-min-lasting sudden onset of high levels of action potentials. Regardless of the substrate, SNA was significantly lower at hypoxia (A two-way ANOVA with Bonferroni correction). There were minor but statistically not significant (multiple *t*-tests with Sidak–Bonferroni correction for multiple comparisons) differences of relative activity levels between seal slices kept in 10 mM glucose ($n = 9$ slices) and slices kept in 20 mM lactate ($n = 10$ slices) (Fig. 4). After 60-min hypoxia and 20-min reoxygenation, the average relative activity level was 40 ± 47% (median = 22.2%, range 0 to 148.7%) in glucose and 22 ± 24% in lactate (median = 14.3%, range 0 to 69.7%). The survival rate, where SNA could be observed for at least 20 min after reoxygenation, was 56% in glucose and 40% in lactate. In mice ($n = 8$ slices) the average time from onset of hypoxia and the irreversible loss of activity was 6.2 ± 3.7 min in glucose, which corresponds well to previous results (Folkow et al., 2008).

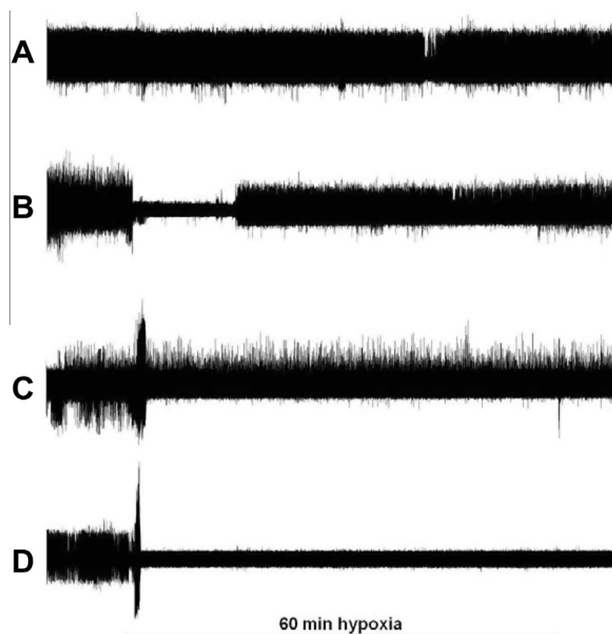


**Fig. 3.** Typical recordings of four types of spontaneous neuronal activity (SNA) changes in response to 60-min hypoxia in 10 mM glucose and 20 mM lactate in isolated cortical slices from hooded seals. (A) Maintained activity throughout normoxia, hypoxia and reoxygenation. (B) Partial loss of activity with recovery in hypoxia. (C) Maintained activity with a sudden onset of high levels of action potentials at the start of hypoxia. (D) Loss of activity in hypoxia without recovery. Recordings from slices with glucose or lactate were very similar, A and B were obtained in glucose, C and D in lactate.

### Responses of mouse and seal brain neurons to aglycemia and ischemia

During and after a 60-min exposure to glucose-free medium, slices from the seal visual cortex ($n = 4$) consistently displayed SNA and remained apparently unaffected by the treatment, except for a surge of activity after ~15 min (Fig. 5). By contrast, in mouse slices ($n = 6$) SNA consistently disappeared without subsequent recovery after 12.17 ± 3.88 min in aglycemia. At 60-min ischemia (simultaneous exposure to hypoxia and aglycemia), seal slices ($n = 4$) displayed a similar response as under hypoxia in glucose and lactate. The average relative activity level was 26 ± 24% (median = 24.5%, range 2–54%) with a survival rate of 50% after 20-min normoglycemic reoxygenation. In mice ($n = 6$ slices) activity was irreversible lost after 3.67 ± 0.52 min in ischemia (Fig. 6).

### Brain glycogen levels

Glycogen measurements revealed an on average threefold higher glycogen content in the hooded seal cortex (gray matter) and cerebellum compared to mouse (Fig. 7). Glycogen levels in the seal ($n = 3$) ranged from 2.3 to 6.4 (mean = 4.1) ng/μg protein in the cortex and 1 to 6.3 (mean = 3.5) ng/μg protein in the cerebellum. The range in mice ($n = 5$) was 0.3 to 1.8 (mean = 1.0) ng/μg protein for the cortex and 0.4–1.9 (mean = 0.9) ng/μg protein for the cerebellum. Due to large variances in seal glycogen levels the differences between seal and mouse did not quite reach statistical significance in the cerebellum, but was significant in the cortex ($p < 0.05$).

### Quantitative analysis of PYGB-mRNA levels

Neocortical and cerebellar levels of PYGB-mRNA in hooded seal and mouse were compared by using quantitative RT-PCR (qRT-PCR). PYGB expression levels in seal and mouse cortex were similar and no significant differences were found. In contrast, mouse cerebellum showed 1.6-fold ($p = 0.007$) higher PYGB levels compared to the seal cerebellum. While PYGB-mRNA copy numbers were found to be very similar in the cortex and cerebellum of the hooded seal, the mouse cerebellum presented more than twofold higher expression levels than the cortex ($p = 0.0014$) (Fig. 8).

## DISCUSSION

The remarkable hypoxia-tolerance of brain neurons of the hooded seal (Folkow et al., 2008; Ramirez et al., 2011) requires explanation and has stimulated our research. Because the brain slices from seals and mice had the same thickness and were kept under same conditions, the tolerance of the seal neurons cannot be attributed to differences in blood flow, vascularization, blood respiratory properties or temperature effects. Thus, there must be intrinsic factors and adaptations of the cellular metabolism that help the seal's neurons to endure hypoxic periods. These may include modified biochemical pathways, specific protective mechanisms or additional energy

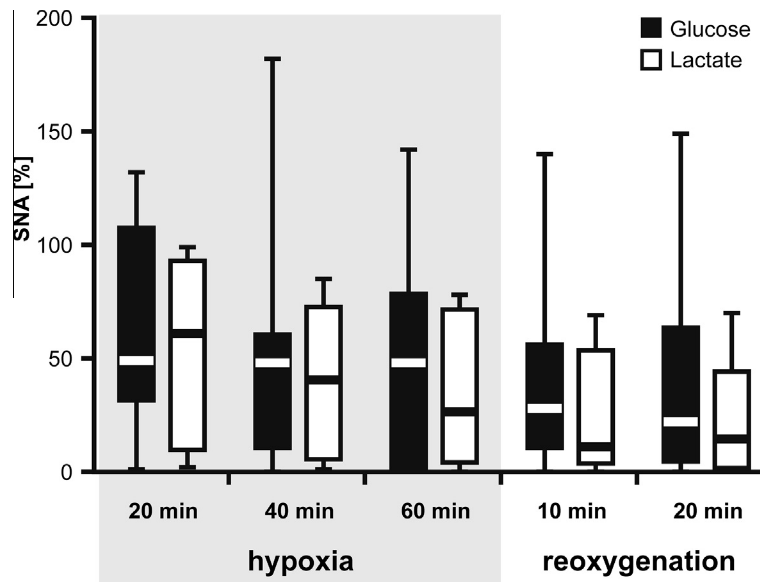


**Fig. 4.** Relative spontaneous neuronal activity (SNA) in isolated hooded seal cortical slices during 60-min hypoxia and 20-min reoxygenation in 10 mM glucose (black; $n = 9$) or 20 mM lactate (white; $n = 10$). Control SNA was averaged for 10 min in normoxia prior to start of hypoxia and was set to 100%. Medians, interquartile ranges and minima, and maxima are given.

stores, but also interactions between neurons and glia cells.

## Seal neurons do not discriminate between lactate and glucose, but are tolerant to lactate

In a previous study, we found that in seal brains markers of aerobic metabolism mostly reside in astrocytes, while in terrestrial mammals these enzymes are in neurons (Mitz et al., 2009; Schneuer et al., 2012). This was interpreted in terms of a reverse ANLS, in which seal neurons mainly consume glucose anaerobically, with the produced lactate transported to and metabolized aerobically in astrocytes. According to the ANLS hypothesis, neurons of a ''normal'' brain work largely aerobically and prefer lactate, produced by astrocytes (Itoh et al., 2003; Pellerin, 2005). Lactate provides a ready-to-use aerobic energy substrate with no initial ATP cost, which also enhances neuronal function during the initial stages of reoxygenation when both ATP and glucose pools are exhausted (Schurr et al., 1997c).

Here, we compared spontaneous extracellular neuronal population activity of the visual cortex in hooded seals and mice during 1-h hypoxia and 20-min reoxygenation in aCSF with either 10 mM glucose or 20 mM lactate. Our data revealed in seal neurons no significant differences of relative levels of SNA with the two substrates during normoxia (Fig. 2). During hypoxia and at recovery, the median levels of SNA were slightly higher in glucose than in lactate and appeared to decrease during incubation, but the differences were not significant (Fig. 4). The lack of significance in these measurements is probably due to the large standard deviations.

In any case, seal neurons survive incubation in lactate and remained active. This finding is surprising, since elevated lactate is known to suppress neuronal firing *in vivo* and *in vitro* (Gilbert et al., 2006). Indeed, this is what we observed in mouse neurons, although e.g. Schurr et al. (1988) reported survival and recovery of rat neurons in 20-min lactate. However, under conditions, which differ in terms of pre-incubation times, SNA disappeared from mice neurons treated in lactate after 15 min already at normoxia. This might be interpreted either in terms of lower glycogen stores, which makes constant supply of glucose to the mouse brain necessary even at normoxia (see below), or might be due to lactic acidosis. Although mild lactic acidic conditions during hypoxia and reoxygenation have a protective effect against neuronal damages, severe lactic acidosis can be deleterious (Schurr, 2006; Dienel, 2012). As we focused on differences in neuronal response to glucose and lactate in the seal we did not trace the pH of brain slices.

The observed tolerance of neurons from the hooded seal brain to lactate is remarkable. Lactate accumulation in seals particularly occurs at the end of long dives and may reach up to 14 mM in the blood plasma (Kerem and Elsner, 1973; Hochachka et al., 1977; Hochachka, 1981; Davis, 1983; Elsner et al., 1989; Ponganis, 2011). In addition, even under resting conditions 20–25% of the utilized blood glucose was released as lactate, whereas in rats it was only 5–15% (Murphy et al., 1980). Thus, even if lactate is not a preferred substrate of seal neurons, their ability to tolerate high concentrations is likely an adaptation to the diving lifestyle. We also hypothesize that the ability to maintain activity in the absence of glucose, in the presence or absence of lactate, is due to the enhanced glycogen store in the seal brain (see below).

Also the proportions of neocortical seal slices displaying either maintained activity with survival, recovery after silence or no survival were not significantly different. These different responses shown in both substrates (glucose or lactate) suggest that neurons employ different strategies to survive hypoxic

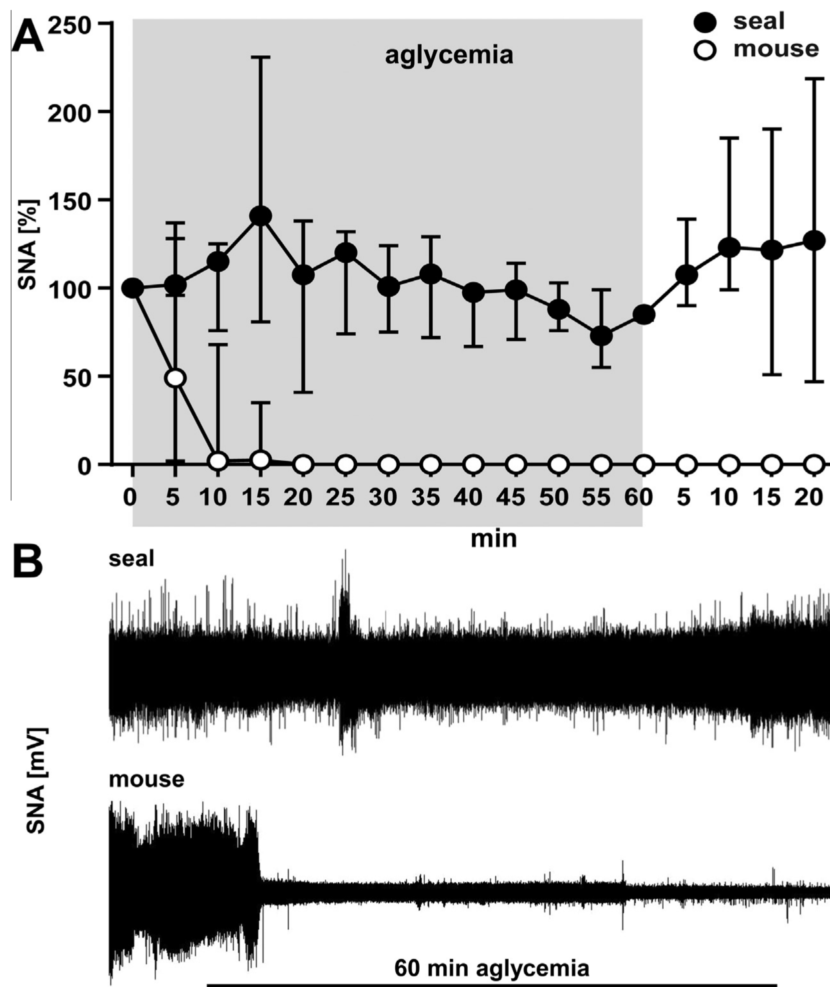


**Fig. 5.** Spontaneous cortical neuronal activity (SNA) in response to 60-min aglycemia followed by 20-min normoglycemia (10 mM glucose). (A) Relative SNA levels in hooded seal (black circles; $n = 4$) and mouse (white circles; $n = 6$). Control SNA was averaged for 10 min in normoglycemia prior to start of aglycemia and was set to 100%. Medians with minima and maxima are given. (B) Typical recordings in isolated slices from hooded seal and mouse.

insult. The suppression of neural activity with subsequent recovery after reoxygenation may represent a protective shut-down to reduce neuronal requirements for ATP. As diving seals must remain active and alert, Folkow et al. (2008) assumed that hooded seals employ a selective neuronal hypometabolism, but with some cerebral networks continuing to control vital functions (Ramirez et al., 2007). Similar observations of differential neuronal responses to hypoxia have also been made in an avian diver, the eider duck, *Somateria mollissima* (Ludvigsen and Folkow, 2009).

In agreement with previous results obtained under slightly different conditions (Folkow et al., 2008), mouse neurons were found to be less well adapted to hypoxia than those of seals and endured only about 6 min of hypoxia.

## Higher intrinsic glycogen stores and other adaptations support hypoxia tolerance of the seal's brain

The enhanced hypoxia tolerance of the seal brain and some other evidence suggests that it possesses a high capacity for anaerobic metabolism (Lenfant et al., 1969; Kerem and Elsner, 1973; Hochachka, 1981). We therefore investigated the aglycemic (no glucose) and ischemic (no oxygen, no glucose) response of hooded seal neurons (Figs. 2 and 3). SNA remained essentially unaltered during 1 h of aglycemia. During and after return from ischemic conditions to normoglycemic reoxygenation, the response and survival rate did not statistically differ from slices superfused with glucose or lactate in hypoxic conditions. This provides further evidence that seal brain neurons are able to abide neuronal activity for more than 1 h without an external energy supply and suggests that they have to use an alternative energy source inherent to neurons or brain tissue.

We therefore assumed that the seals' brains must have a high glycolytic capacity. In fact, compared to mice we found about fourfold higher levels of glycogen in the brain of the hooded seal (Fig. 7). This agrees with previous studies with the Weddell seal (*Leptonychotes weddellii*), which showed that that brain glycogen levels are in fact 2–3 times higher in this species compared to non-diving mammals (Kerem et al., 1973). In adult terrestrial mammals glycogen is present only in astrocytes

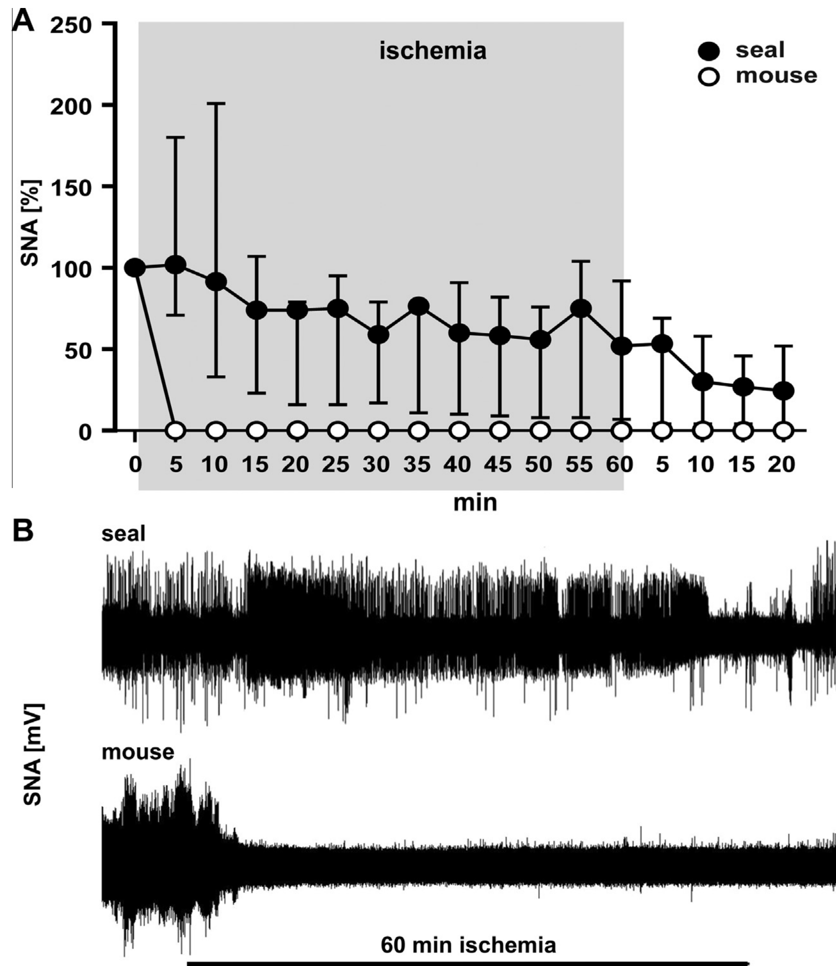


**Fig. 6.** Spontaneous neuronal activity (SNA) in response to 60-min ischemia and 20-min normoglycemic reoxygenation. (A) Relative spontaneous activity levels in hooded seal (black circles; $n = 4$) and mouse (white circles; $n = 6$). Control SNA was averaged for 10 min in normoxia/normoglycemia prior to start of ischemia and was set to 100%. Medians with minima and maxima are given. (B) Typical recordings in isolated cortical slices from hooded seal and mouse.

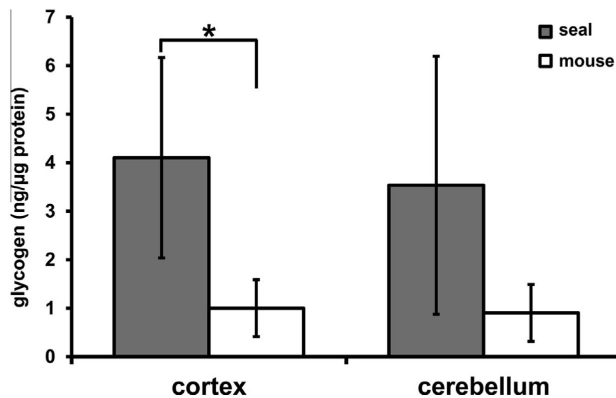


**Fig. 7.** Mean ± range glycogen content in the cortex and cerebellum of hooded seal ($n = 3$) and mouse ($n = 5$). *Statistically significant difference ($p < 0.05$).

(Cataldo and Broadwell, 1986; Brown and Ransom, 2007). We hypothesize that in seals this increased glycogen content is due to the expression of glycogen in neurons. The situation may be similar to the situation in terrestrial embryos in which glycogen is expressed also in neurons (Bloom and Fawcett, 1968), a developmental stage which has been associated with higher metabolic demand or less secure glucose supply (Brown and Ransom, 2007) and also enhanced neural hypoxia tolerance.

Although the glycogen stores in the brain are lower than those found in skeletal muscles (Kerem et al., 1973; Henden et al., 2004), the elevated brain glycogen stores may provide an important energy supply for the maintenance of anaerobic glycolysis in the seal's brain, and it may confer neuroprotection. Brain glycogen is thought to provide the energy substrate under conditions when blood-borne glucose becomes insufficient for example during transient elevations in energy demand (Brown et al., 2003; Choi et al., 2003). Brain glycogen metabolism is known to be affected by blood glucose levels (Choi et al., 1999, 2003). Under normal conditions plasma glucose levels are tightly controlled by the interplay between the liver, muscle, pancreas and brain. However, this interaction becomes defective in the context of diabetes. Indeed a single episode of insulin-induced hypoglycemia is sufficient to dysregulate the interplay between the liver,

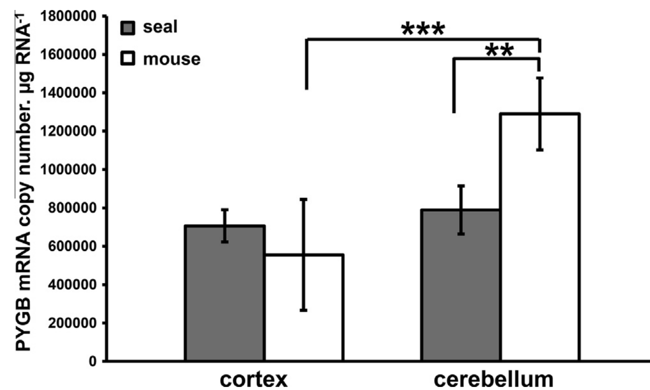


**Fig. 8.** Quantification of Pygb mRNA expression (means ± SD) in the cerebral cortex and cerebellum of hooded seal ($n = 3$) and mouse ($n = 5$). Significance levels, as indicated by asterisks: $^{***}p < 0.001$, $^{**}p < 0.01$.

muscle, pancreas and brain (Veneman et al., 1993). These hypoglycemic events lead to upregulation of brain glycogen concentrations (Gruetter, 2003), not only in diabetes, but also under hypoxic ischemia (Brucklacher et al., 2002). Increased brain glucose levels are thought to be neuroprotective (Swanson and Choi, 1993; Choi and Gruetter, 2003). Thus, in seals, enhanced glycogen stores may be more important than in terrestrial mammals, as in these animals blood glucose undergoes more drastic shifts – under their normal physiological conditions. Indeed, it has been suggested that seals represent naturally occurring diabetic animal models, in part due to their elevated blood glucose levels (Bennett et al., 2013). Thus, blood glucose levels in seals are generally well maintained, even throughout long dives (Guppy et al., 1986). This suggests that blood glucose depletion is not a factor that limits diving capacity in seals.

To analyze the glycogen degradation rate we measured the mRNA levels of Pygb, an enzyme that is assumed to catalyze the rate-determining step. Pygb mRNA levels were slightly lower in the seal brain than that in mice (Fig. 8). This may indicate that glycogen degradation rates may not differ. Thus, rather the glycogen content itself may matter in hypoxia adaptation. However, it must be considered that *i.* mRNA levels are at best a proxy of the actual protein levels and that *ii.* enzymatic activities may actually be different. Also, previous studies using other techniques have demonstrated an enhanced enzymatic capacity for anaerobic glycolysis in diving species (Messelt and Blix, 1976; Shoubridge et al., 1976), including seals (Murphy et al., 1980).

## CONCLUSION: THE ENERGY METABOLISM IN THE SEAL'S BRAIN

In agreement with previous results (Ramirez et al., 2007, 2011; Folkow et al., 2008), this study shows that the brain neurons of the hooded seal display a high hypoxia tolerance. Here we show that the neurons of the hooded seal are also more tolerant toward lactate and changes in exogenous substrate availability. Both hypoxia- and lactate tolerance can be considered as an adaptation of the seal's brain to long dives, during which oxygen levels in blood and tissue decrease and blood lactate levels may increase. Higher intrinsic glycogen stores may be protective and enhance the anaerobic and ischemic survival of the brain neurons, but it remains uncertain whether these adaptations alone are sufficient to explain the hypoxia tolerance. A reverse ANLS in the hooded seal brain may also be instrumental in enhancing hypoxia survival of neurons (Mitz et al., 2009; Schneuer et al., 2012). This mechanism would require significant glycolytic activity of the neurons. Although we found higher glycogen content in the gray matter of the seal cerebrum, we cannot conclude that this energy store resides in the neurons.

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