Neuroscience 275 (2014) 374–383

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

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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.**

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

http://dx.doi.org/10.1016/j.neuroscience.2014.06.024
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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

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

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