

Faculty of Biosciences, fisheries and Economics

Department of Arctic and Marine Biology

Efflux of neurotransmitter from brain slices during O₂ deprivation – comparing diving and non-diving species.

Lorenzo Ragazzi

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Supervisor

Professor Lars P. Folkow, UiT The Arctic University of Norway

Co-Supervisor

Samuel J. Geiseler, UiT The Arctic University of Norway



Cover picture: Females of common eider duck (*Somateria mollissima*).
Photo: Erling S. Nordøy

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Abbreviations:

Ala	Alanine
Arg	Arginine
Asp	Aspartate
CNS	Central nervous system
Cys	Cysteine
Glu	Glutamate
Gly	Glycine
Ile	Isoleucine
IS	Internal Standard
Leu	Leucine
Lys	Lysine
Met	Methionine
NVa	Norvaline
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
Val	Valine

Abstract

During hypoxic insult, neuronal tissue undergoes an excitotoxic cascade of events leading to cell death. This response involves loss of membrane potential and uncontrolled release of primarily excitatory neurotransmitters.

I propose that this fatal cascade is significantly delayed or attenuated in diving species, which are adapted to repeated exposures to hypoxic conditions as a consequence of breath-hold diving. Here I have compared the in vitro overflow/efflux of neurotransmitters from cerebellar slices of a diving bird, the common eider duck (*Somateria mollissima*) and of the non-diving chicken (*Gallus gallus domesticus*), using a microperfusion technique as described by Kirschner et al. (2009).

Slices were cut in ice cold oxygenated aCSF and stored at room temperature before transfer to custom-made microperfusion chambers. After 20 min equilibration at 37°C, the superfusate was collected while exposing the slices to 20 min hypoxic or normoxic (control) perfusions, before reoxygenation. I used Waters UPLC AAA application solution for physiological amino acids to detect changes in the concentration of the excitatory neurotransmitters Glutamate and Serine and the inhibitory Glycine, in the superfusate. I found that neurotransmitter release, for both eider duck and chicken, tended to increase in hypoxia. In addition, higher levels of neurotransmitter efflux during the hypoxia, and normoxia exposure were present in the chicken.

1. Introduction

Marine mammals and seabirds live in a challenging environment. They spend most of their life in the sea and under water. The diving feats of pinnipeds and cetaceans are well known. Research over decades has revealed a good picture about the diving physiology of marine mammals (Snyder, 1983b, Kooyman and Ponganis, 1998, Ramirez et al., 2007, Goldbogen et al., 2013). Recent studies confirm also that many species of seabirds are able to handle protracted and deep dives (Johansson and Aldrin, 2002, Wright et al., 2014). Thanks to physiological adaptations that have evolved over time, marine mammals like the hooded seal (*Cystophora cristata*) (fig.1.1) and seabirds like the emperor penguin (*Aptenodytes forsteri*), can hold their breath respectively for more than 50 and 10 minutes and dive respectively to more than 1000 and 400 m depth (Folkow and Blix, 1999, Wright et al., 2014).



Figure 1.1 – Blueback, hooded seal (*Cystophora cristata*) when it is up to about 14 months of age. In the West Ice.
Photo: Lorenzo Ragazzi

1.1 Diving physiology

Oxygen storage

When diving, animals break contact with the atmosphere, therefore they need to carry O₂ with them to enable tissues to function aerobically.

Marine vertebrate are able to store oxygen in higher concentrations than land vertebrates. The blood store of terrestrial mammals is about 14-15 ml O₂ per kg of body mass (Costa, 2001), while

in seabirds like penguins and diving mammals like hooded seals, it may reach 33 ml O₂/kg to 50 ml O₂ / kg respectively, two to five times higher (Costa, 2001, Burns et al., 2007).

Because of high myoglobin concentrations, the skeletal muscles of some diving mammals appear almost black. In humans, skeletal muscles contain about 4-5 mg of myoglobin per gram wet weight (Möller and Sylvén, 1981). Hooded seals have 70-90 mg of myoglobin per gram wet weight (Burns et al., 2000, Lestyk et al., 2009). Diving birds also, like penguins and ducks, showed high myoglobin concentration, 4-64 mg per gram wet weight depending of the species (Weber et al., 1974, Stephenson et al., 1989, Ponganis et al., 1999).

In relation to the blood oxygen storage and the cardiovascular system, the spleen plays an important role releasing red blood cells in the circulatory system during diving. In Weddell seals (*Leptonychotes weddellii*) it was measured a difference of 14% red blood cells (by volume) when the animal is resting and when the animal is diving (Qvist et al., 1986). Cabanac (2000) estimated that in hooded seals the splenic blood oxygen store may contribute by about the 9% of the total body oxygen store (Cabanac, 2000). An elevated red blood cells concentration increases the viscosity of the blood, the spleen has the role to partly store the red blood cells when the animal is not diving.

The lungs of many shallow-diving species, such as seabirds, sea otters and some otariids, is an important O₂ store (Costa, 2001). By contrast, deep-diving species normally exhale before diving, and their lungs collapse (Costa, 2001). This reflects a lack of support on lung O₂ stores but also the need to avoid decompression sickness and reduce buoyancy during diving (Ramirez et al., 2007).

Cardiovascular responses

During short voluntary dives, the circulatory system remains open to almost all body regions and the tissues receive, from the body's blood O₂ storages, enough O₂ to metabolize aerobically (Hill et al., 1989). Contrary, the O₂ store is inadequate to sustain a constant supply of O₂ during prolonged dives and such demand is difficult to satisfy.

Throughout long-duration diving, in seals (Zapol et al., 1979) and ducks (Johansen, 1964), blood flow to most tissues is mostly reduced by vasoconstriction. The main portion of the stored O₂ is reserved for the central nervous system (CNS hereafter) and heart, which in part dependent on aerobic catabolism for synthesis of ATP. The other tissues can turn to anaerobic metabolism, accumulating lactic acid if the dive is prolonged (Scholander, 1940). High blood buffering

capacities, in marine mammals, avoid pH falling in tissue and blood due to the increase of CO₂ and lactic acid (Snyder, 1983a).

Because of the widespread vasoconstriction, the heart rate decreases, sometimes dramatically. Substantial diving bradycardia was first demonstrated during simulated diving in seals, by Scholander (1940), and has later been observed also during voluntary diving in a range of species, including gray seals (*Halichoerus grypus*) (Thompson and Fedak, 1993), harbor seals (*Phoca vitulina*) (Jobsis et al., 2001), emperor penguins (*Aptenodytes forsteri*) (Wright et al., 2014) and common eider duck (*Somateria mollissima*) (Hawkins et al., 2000).

The massive peripheral vasoconstriction shuts down blood supply to most tissues. The blood PO₂ drops and in the brain, the blood supplied has a very low oxygen content, eventually, resulting in a severe hypoxemia (low blood oxygen content insufficient to sustain metabolic processes) (Meir et al., 2009). The critical arterial oxygen tension (the minimum amount of dissolved oxygen necessary to sustain aerobic metabolic processes) for (non-diving) mammals is 35-40 mmHg (Erecinska and Silver, 2001). Northern elephant seal (*Mirounga angustirostris*) have shown that cerebral integrity is maintained down to 23 mmHg (Meir et al., 2009) and in emperor penguins as low as 22 mmHg during dives (Ponganis et al., 2007).

In diving animals, neither heart nor brain are entirely dependent on aerobic metabolism. Studies revealed that myocardial glycogen content, in isolated harp seal cardiomyocyte, is approximately 10 times more than that of rats (Henden et al., 2004). In addition, during ischemia lactate production is significantly greater in seal compared to rat cardiomyocyte. Cellular ATP is well maintained and Ca²⁺ is not affected in ischemic seal cardiomyocyte, while in rat ATP 65% decline and Ca²⁺ increase (Henden et al., 2004). In harbour seals (*Phoca vitulina*), during simulated diving, the lactate levels rise in the venous effluent from their head towards the end of the protracted dive, when the arterial blood O₂ tension drops below 25 mmHg (Kerem and Elsner, 1973). Recordings from neocortical slices have also shown that these may maintain activity for up to 60 min in hypoxia (Ramirez et al., 2011). Likewise, was recorded spontaneous activity for extended durations in cerebellar slices of eider ducks during induced anoxia (Ludvigsen and Folkow, 2009). The brain's dependence on anaerobic metabolism increases as the animal becomes hypoxic. The cardiac output in spotted seals (*Phoca largha*) and grey seals (*Halichoerus grypus*) during experimental diving decline 90% in the first 2-5 min of dive (Blix et al., 1983). Cerebral cortical and adrenal flow decrease and liver, kidney, fat, skin, and stomach became ischemic throughout the dive (Blix et al., 1983).

Hypometabolism

Hypometabolism, first conceptualized by Scholander (1940), is an important strategy to cope with hypoxia. When a diving mammal or bird dive, there is an overall reduction in metabolism. This hypometabolic state, reduce the O₂ requirements and consequently avoid the effects of hypoxia (Costa, 2001, Larson et al., 2014).

1.2 Physiology of the diving brain

Although many physiological adaptations of marine mammals and seabirds have been reported, little is known about the mechanisms that sustain the neuronal activity in the brain and how it can sustain the high demands for metabolic energy (O₂ supply) during long hypoxic periods.

Electrophysiological studies have shown that isolated cerebral neurons of adult hooded seal (*Cystophora cristata*) appear to exhibit an intrinsic hypoxia tolerance and that they can survive much longer at low oxygen levels than those of mice (Folkow et al., 2008). Similar studies using extracellular recordings of spontaneous activity in cerebellar slices from eider duck (fig. 1.2), chicken and rat, showed that avian neurons are more tolerant to hypoxia exposure than rat neurons and that eider duck neurons are even more hypoxia-tolerant than chicken neurons (Ludvigsen and Folkow, 2009), but the underlying mechanisms remain to be elucidated.

Brain temperature measurements have shown that seals respond to experimental short dives with a controlled cooling of their brain, with a significantly decrease of 2°C in harp seals and 3°C degree in hooded seals (Odden et al., 1999, Blix et al., 2010). This observation suggested that these animals, may reduce their cerebral oxygen requirements by 25%, to extend their diving capacity (Blix et al., 2010). Which is one of the mechanisms by which hypometabolism can be achieved. A similar adaptation was recorded also in Pekin ducks (*Anas platyrhynchos domestica*), which during simulated dives displayed a drop of the hypothalamic temperature of 3°C (Caputa et al., 1998).



Figure 1.2 – Females of common eider duck (*Somateria mollissima*). Photo: Erling S. Nordøy

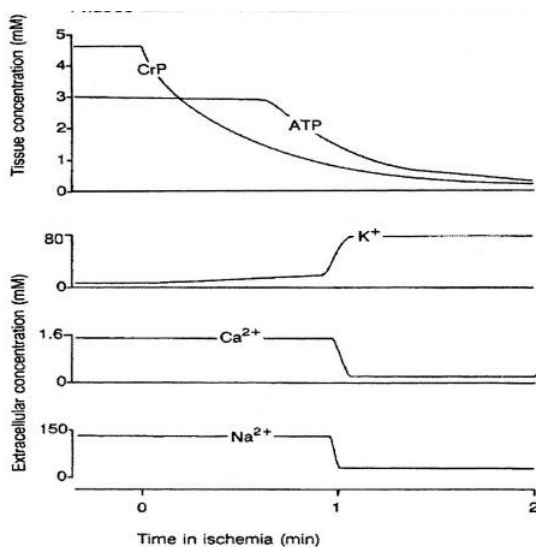
According to Glezer et al. (1987) and Kerem & Elsner (1973) some cerebral protection to hypoxemic challenge, is maybe offered by the high brain capillary density found in whales and seals, which reduce the diffusion distance and improve the flow of O₂ to neurons (Kerem et al., 1973, Glezer et al., 1987).

An analogous of the oxygen-binding proteins hemoglobin and myoglobin, namely neuroglobin, which is predominantly expressed in the brain, was discovered by (Burmester et al., 2000). Today it is thought that the neuroglobin may play a key role for maintenance of aerobic metabolism in neural tissue by facilitating O₂ diffusion. Furthermore this protein also has a detoxifying action of reactive oxygen species (ROS) (Burmester and Hankeln, 2009), which are generated during and after diving (Zenteno-Savin et al., 2002). Whales, have been shown to have neuroglobin mRNA expression levels that are 4 to 15 times higher than in seal, cow (*Bos taurus*) and ferret (*Mustela putorius furo*) brains (Schneuer et al., 2012). However, somewhat surprisingly, in seals cerebral neuroglobin levels are not higher than those of terrestrial mammals (Mitz et al., 2009). Nevertheless, a different distribution with higher levels in glial cells (astrocytes) than in neurons has been demonstrated, based on which it has been postulated that in seals, glial cells are probably more involved in aerobic metabolism than are neurons, which instead are assumed to have a high anaerobic capacity (Mitz et al., 2009, Schneuer et al., 2012). This neural based respiratory protein was also identified in chicken (Kugelstadt et al., 2004), however its distribution and levels in brains of diving birds is not known.

1.3 The anoxic brain

Approximately 20% of the oxygen consumed by the resting human body is used by the brain (Bélanger et al., 2011). The greater part of this oxygen is used to produce the ATP, from glucose oxidation in the mitochondria, required to maintain the membrane potentials essential for electrical signaling with synaptic and action potentials. A lengthy interruption of the oxygen supply to the brain leads to irreversible neurological damage and neuronal death. The hippocampus neurons, and the Purkinje cells of the cerebellum seem to be among the most vulnerable neurons in relation to ischemic damage (Lutz et al., 2003).

Without sufficient ATP production, ATP-dependent neuronal processes including ion transport cease, leading to efflux of K^+ ions and inflow of Ca^{2+} and Na^+ (fig. 1.3). Neurons consequently depolarize, followed by an uncontrolled release of excitotoxic neurotransmitters (e.g. Glutamate, Serine) (Lutz et al., 2003). High extracellular levels of excitatory neurotransmitters are cause of ischemic neuronal damage (Lutz et al., 2003). In particular glutamate plays an important role as an agonist of both the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (also known as AMPA receptor) the N-methyl-D-aspartate receptor (also known as NMDA receptor) (Lutz et al., 2003). The activation of both those receptors, allows an increase of intracellular Ca^{2+} levels with



devastating consequences. The rise of Ca^{2+} levels, acts as a trigger for the activation of enzymes such as lipases, endonucleases and proteases (Lutz et al., 2003). D-ser is a coagonist (Shleper et al., 2005), necessary to activate the NMDA receptor and contribute to this so-called excitotoxicity (Kirschner et al., 2009).

Figure 1.3 – Changes in creatine phosphate (CrP), ATP and extracellular ions concentration during ischemia in the rat brain (Lutz et al., 2003).

1.4 Introduction of the methodology used

Previous studies, gave already some information about the dynamic efflux of excitatory (e.g. Glu, Ser) and inhibitory (e.g. Gly) neurotransmitters during cerebral ischemia. According to Kirschner et al. (2009), Glu is released within a few minutes after the onset of hypoxia, an early event in the neurotoxic cascade (Kirschner et al., 2009). Ser was suggested to contribute to excitotoxicity in

several brain regions as a coagonist of the NMDA receptor (Katsuki et al., 2004). Gly, is well established as an inhibitory neurotransmitter in lower brain areas (Lutz et al., 2003). It is an antagonists of the D-ser ("Gly") binding site, neuroprotective in animal models of stroke (Wasterlain et al., 1996, Ohtani et al., 2000, Ohtani et al., 2003). When inhibitory transmitters are released in large amounts (in hypoxia tolerant species), they act by depressing neural activity and hence ATP use and thereby protect (Nilsson, 1990, Nilsson et al., 1990).

In hippocampus slice of rat, Thr levels increase during oxygen deprivation (OD) (Kirschner et al., 2009). According to Kirschner (2009), the chemical similarity between Thr and Ser suggests that these two amino acids use similar transport mechanisms.

Hypoxia-tolerant species as the turtle (*Pseudemys scripta elegans*) (Nilsson et al., 1990), the crucian carp (*Carassius carassius*) (Nilsson, 1990) and the arctic ground squirrels (*Urocitellus parryii*) (Ross et al., 2006, Drew et al., 2013), have shown a blunted excitotoxic response to hypoxia. However, no study has been conducted on diving birds and mammals.

In the present study, single cerebellum slices have been used to monitor temporal efflux of Glu, Ser, Gly and Thr in response to oxygen deprivation in a diving and in a non-diving bird, the eider duck and the chicken.

1.5 Objectives and hypothesis

With this study I would like to test the hypothesis that the fatal hypoxia-induced excitotoxic cascade is significantly blunted also in diving species, which are adapted to repeated exposures to hypoxic conditions as a consequence of their breath-hold diving during feeding. In order to test this hypothesis I used a modified version of the approach used by Kirschner et al. (2009), to compare the in vitro temporal profile of efflux of neurotransmitters that play a crucial roles in the excitotoxicity cascade, from isolated brain slices during normoxic and simulated hypoxia conditions, in both a diving and a non-diving species. As a model organism I used the common eider duck (*Somateria mollissima*) and the chicken (*Gallus gallus domesticus*). The same experiment was also performed on hooded seals, however no results are available yet for this species or from a control (non-diving) mammalian species, due to time and analyzing capacity limitations.

Research on neuronal adaptation to hypoxia of diving species might increase our knowledge of how the nervous system is adapted for life in specific ecological niches. Also this information could contribute to develop/improve therapies towards neurological anomalies such as stroke.

2. Materials and methods

2.1 Animal handling

All animal handling was conducted in the research animal facilities at the Arctic Biology building (ABB hereafter) of UiT The Arctic University of Norway. Samples have been collected from six eider ducks (*Somateria mollissima*) (tab. 2.1), which originated from birds collected as eggs on Grindøya outside Tromsø and reared in the research animal facilities at the ABB, and four chickens (*Gallus gallus domesticus*) (tab. 2.2), hatched and reared in similar conditions by a private owner (Professor Erling S. Nordøy). A suitable fenced outdoor area with a small pool, during the summer and an indoor facility, also with a pool in the winter season, ensured the well-being of the eider ducks. Samples have been collected also from eight hooded seals (*Cystophora cristata*). However, because of time limitations, the results from studies of the latter have not been completed and will therefore not be shown in this thesis. All experiments were conducted in accordance with current Norwegian legislation (The Animal Welfare Act and Regulations for use of animals in research (Forskrift for forsøk med dyr; FOR-1996-01-15-23)).

2.2 Anesthesia & euthanasia

The chickens and the eider ducks were anaesthetized with an intramuscular injection of Ketalar (ketamine hydrochloride 50 mg/ml, Pfizer Inc., New York, USA) and Xylazin (Rompun vet. xylazin chloride 20 mg/ml, Bayer AG, Leverkusen, Germany) (Green et al., 1981, Gales, 1989) before being euthanized by decapitation (tab 2.1.1 and tab 2.1.2).

Chicken ID	Date of the experiment	Age (months)	Sex	Weight [Kg]	50 mg/ml Ketalar + 20 mg/ml Xylazin
160913	16.09.2013	6	♂	1,84	0,5 ml Ketalar + 0,3 ml Xylazin
180913	18.09.2013	6	♂	2,03	0,6 ml Ketalar + 0,3 ml Xylazin
200913	20.09.2013	6	♂	1,95	0,7 ml Ketalar + 0,4 ml Xylazin
230913	23.09.2013	6	♀	1,67	0,6 ml Ketalar + 0,3 ml Xylazin

Table 2.1.1 Data for chickens at the time of the experiment.

Eider duck ID	Date of the experiment	Age (months)	Sex	Weight [Kg]	50 mg/ml Ketalar + 20 mg/ml Xylazin
230813	23.08.2013	12	♀	1,83	0,8 ml Ketalar + 0,4 ml Xylazin
270813	27.08.2013	12	♀	1,89	1,2 ml Ketalar + 0,6 ml Xylazin
290813	29.08.2013	12	♀	2,15	0,9 ml Ketalar + 0,5 ml Xylazin
020913	02.09.2013	12	♂	2,06	1,2 ml Ketalar + 0,6 ml Xylazin
040913	04.09.2013	12	♂	2,20	1,0 ml Ketalar + 0,5 ml Xylazin
060913	06.09.2013	12	♀	2,00	1,2 ml Ketalar + 0,6 ml Xylazin

Table 2.1.2 Data for the eider ducks at the time of the experiment.

2.3 Preparation of Artificial Cerebrospinal Fluid (aCSF)

Neuron cells produce ATP with glucose and oxygen to maintain their metabolic processes and to keep their ionic gradient across the cell membrane. The aCSF mimics the extracellular environment and allows neurons to survive in vitro for at least 12 hours (Wang and Kass, 1997, Sveistrup, 2005).

Compound	mM	g / 10 l
NaCl	128	74.8
KCl	3	2.2
NaH ₂ PO ₄ X H ₂ O	0.5	0.6
NaHCO ₃	24	20.15
CaCl ₂ X 2H ₂ O	1.5	2.2
MgCl ₂	1.0	0.95
D(+) Glucose	10	18.03

Table 2.3.1 - Artificial cerebrospinal fluid recipe according to (Ludvigsen and Folkow, 2009).

The final pH of the aCSF was adjusted to 7.4 ± 1 after 20 min saturation with a 5% CO₂ and 95% O₂ gas mixture. The aCSF was kept at 5°C for not more than one week. Although the fluid should be stable for 3–4 weeks according to (Anonymous, 2011).

2.4 Preparation of tissue blocks

After the animal was decapitated the skull was handled inside a bucket filled with ice-cold aCSF. Low aCSF temperature slows down the metabolic rate and allows cells to survive the ischemic period during carrying and dissecting (Wang and Kass, 1997).

Within an average of 5 minutes after decapitation, the brain was removed from the skull and the dissection started. Using a sharp blade, cubes of 1 cm³ tissue were cut out of the cerebellum of the chickens and eider ducks.

2.5 Vibratome method

After dissecting, the tissue cube was fixed onto a stage for slicing (fig. 2.5.1) using cyanoacrylate glue (Loctite® Super Glue, Henkel Corporation, Westlake Ohio) to attach it to the stage. Two different vibratomes (Leica VT1000S, Leica Biosystems, Heidelberg, DE; OTS-3000, FHC Brunswick, ME) were used to prepare brain slices. A solid agar (agumedia, manufacturers, INC. Lansing, Michigan) gel block (6 g in 100 ml of distilled water) was used to prevent the tissue from moving due to the vibratome blade (fig. 2.5.1). The speed was set in such a way that it did not twist the tissue while slicing. The vibratome was adjusted to cut slices 400 µm thick. According to (Wang and Kass, 1997) this thickness secures adequate diffusion of oxygen into the core of the slice. Slicing was performed at a cold temperature of 4°C, both tissue and blade were submerged in ice-cold aCSF aerated with 95% O₂ and 5% CO₂, CO₂ must be present to stabilize the pH to about 7.4 (fig. 2.5.1).

After slicing, each slice was incubated inside a glass chamber in aCSF and aerated with 95% O₂ and 5% CO₂ at room temperature for at least 20 minutes of equilibration. A metal net prevents the slices from sticking to the plexiglass wall (fig. 2.5.2). Electrophysiology recordings (Ludvigsen and Folkow, 2009) suggest that the slices incubated in such an incubation-chamber were able to survive for more than 24 hours.

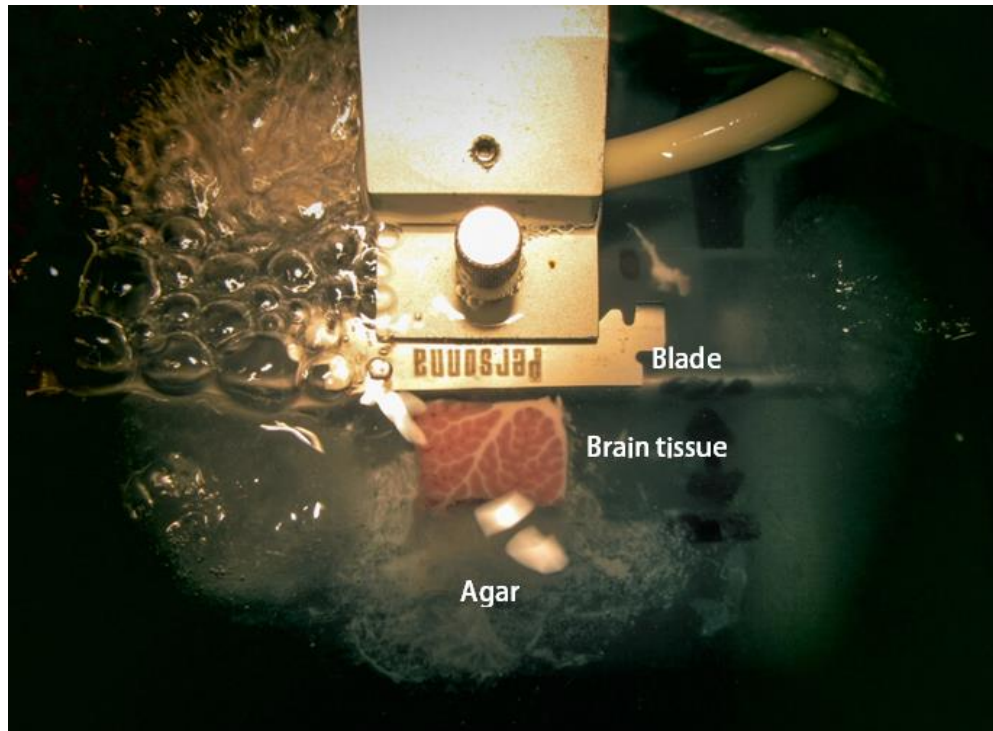


Figure 2.5.1 - Arrangement of the agar and tissue on the slicing stage.

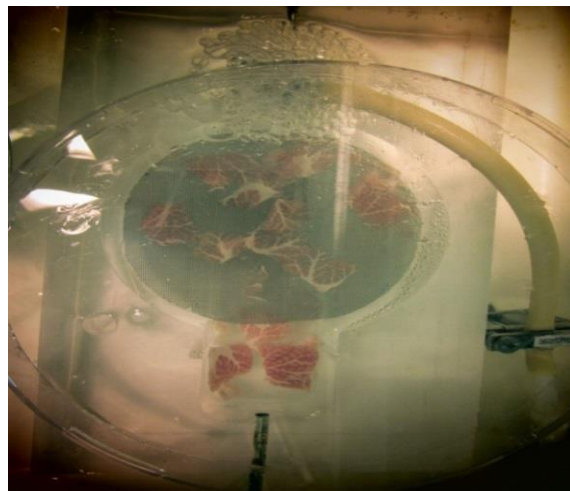


Figure 2.5.2 - The slices were incubated in aCSF at room temperature and aerated with 95% O₂ and 5% CO₂.

2.6 Microperfusion

From the glass chamber, the slices have been transferred one by one, and placed, in a custom-designed homemade brain slice microperfusion chamber, as described by (Kirschner et al., 2009). (The perfusion chamber dimensions are = 1,5 x 1,5 cm wide and 600 μ m deep). Three scientific syringe drivers (Harvard apparatus, Millis, MA; Terumo STC-526, Somerset, NJ) (fig 2.6.2) were used for delivering accurate and precise amount of aCSF (see calibration tests in the Appendix I)

through an apparatus containing three parallel and completely water-tight microperfusion chambers, submerged in a thermostatically heated water bath at 37°C. Slices were perfused at a flow rate of $\approx 0,0234$ ml/min (see Appendix I). The flow rate was dimensioned to the size of slices, based on the information given by Kirschner et al. (2009).

The apparatus containing three parallel microperfusion chambers (two treatments plus one control) was submerged in a thermostatically controlled water bath set at 37°C ($\pm 0,5^\circ\text{C}$), to secure stable temperature conditions. Sampling began after 20 min of slice equilibration to experimental conditions (temperature, perfusion environment) and for stabilization of the neurochemical efflux (Kirschner et al., 2009). Treatments consisted of a 24 min exposure to oxygen deprivation (a much longer period than the dive duration of a eider duck), by perfusing the chambers with aCSF equilibrated with 95% N₂ / 5% CO₂, or to control conditions (perfusion with aCSF equilibrated with 95% O₂ / 5% CO₂). The microperfusion effluent was collected during intervals of 3 minutes to study neurotransmitters temporal response as an in vitro model for simulating diving (see sampling scheme fig. 2.6.1). The 3 min sampling interval was chosen in relation to the perfusion rate and the need for minimum sample size to run UPLC analyses of the constituents of the effluent, in triplicates. The effluent was collected in PCR vials (fig. 2.6.2) and stored at -20°C, until analysis.

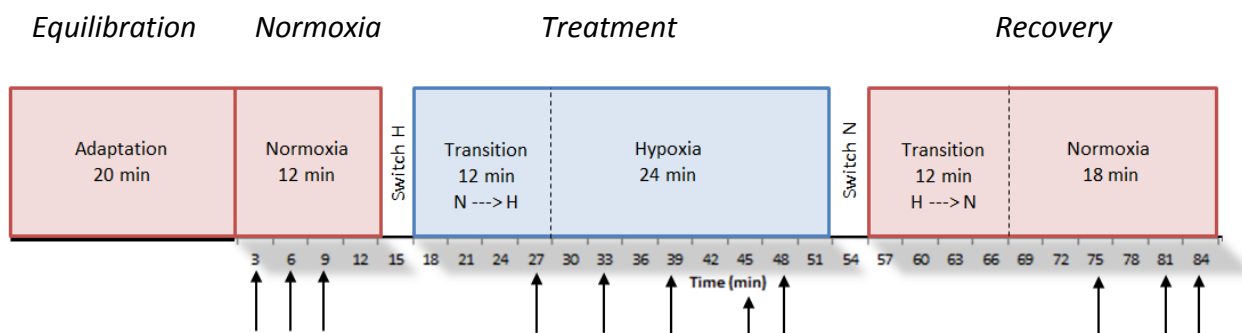


Figure 2.6.1 – Perfusion experiment scheme: Equilibration, normoxia and recovery (aCSF equilibrated with 95% O₂ / 5% CO₂), Treatment (aCSF equilibrated with 95% N₂ / 5% CO₂); H: Hypoxia; N: Normoxia; Black arrows: Analyzed samples.

One should consider that the perfusion experiment might be influenced by factors like flow rate, chamber dimension, lag time between pump and chamber and lag time between chamber and sample vial collector (see perfusion experiment scheme fig 2.6.1). To quantify the lag time factor, the flow profile was monitored using blue methylene in a separated experiment (without a slice). In this way, we could ascertain that 12 minutes were required to let the aCSF flow from the valve

to the microperfusion chamber (fig. 2.6.2). This was taken into account in timing the sampling during the various treatments of the slices, and is referred to as the “transition” in fig.2.6.1.

Due to the employing of syringe pumps (for control, treatment and second treatment as a backup), all of them were adjusted to have the same flow rate (see Appendix I). In order to improve the aCSF flow around the slice within the micro chambers, a titanium net was placed between the bottom and the tissue slice, also small cuts (0.5 mm width) were made on the inner lid of the micro chamber.

At the end of the microperfusion experiments, each cerebellum slice was removed from the microperfusion chamber and weighted.

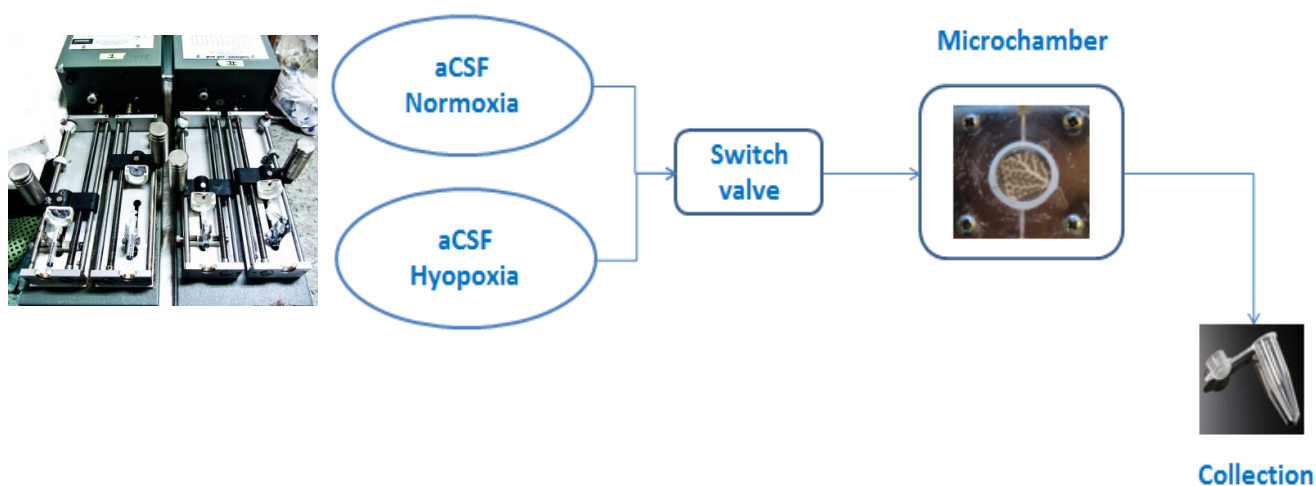


Figure 2.6.2 – On left side four syringe drivers used for perfusion. On right side the perfusion experiment scheme: Treatment (aCSF equilibrated with 95% N₂ / 5% CO₂), adaptation and recovery (aCSF equilibrated with 95% O₂ / 5% CO₂), valve, acute slice inside the micro chamber, collector of the efflux.

2.7 Analysis

2.7.1 General principles for Ultra-Performance Liquid Chromatograph analysis.

All analyses of effluent composition were performed in the Barents Biocentre Lab (Tromsø, Norway), using a Ultra-Performance Liquid Chromatography (UPLC), Waters ACQUITY UPLC H-Class, Waters Corporation, Milford, MA USA.

The Ultra-Performance Liquid Chromatography (UPLC) has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. The principles are shown in fig. 2.7.1:

1 μl of the sample (3) is transferred from the sample vial into a moving fluidic stream, the mobile phase (1). The injected sample is then carried by the mobile phase to the head of a chromatographic column (hydrophobic stationary phase) (4) by a high pressure pump (2) (fig. 2.7.1). The reversed-phase chromatography is based on the principle that hydrophobic molecules in the mobile phase tend to be adsorbed during the hydrophobic stationary phase, and hydrophilic molecules in the mobile phase will pass through the column and are eluted first.

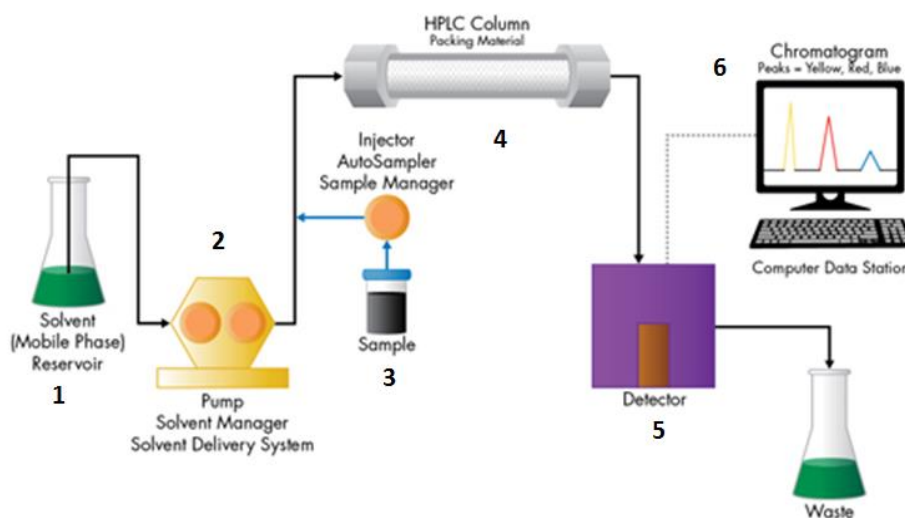


Figure 2.7.1 - Representation of an HPLC/UPLC system (www.waters.com)

Through the column the sample is separated into individual analytes, called “bands”. Each of these bands moves through the column at different speeds, because of the competition between the mobile phase and the stationary phase for attracting each one of the analytes.

As the separated bands leave the column, they pass immediately into a detector (5) (UV detector, Waters ACQUITY UPLC® Photodiode Array (PDA) eλ Detector). Peaks (visible in the chromatogram) are digitally created as an electronic response to the analyte band as it passes through the detector (6) (www.waters.com). A chromatogram is a representation of the separation (4) that has chemically (chromatographically) occurred in the UPLC system. The retention time is the amount of time elapsed from the injection (3) of a sample into the UPLC system to the recording (6) of the peak (band) maximum of the component in the chromatogram (www.waters.com).

Some analytes require derivatization prior to UPLC analysis. The derivatization process makes an analyte more volatile, less reactive, and thus improve its chromatographic performance. Amino acids have polar functional groups and the derivatization replaces active hydrogens (on OH, NH₂,

and SH polar functional groups) with a nonpolar moiety. The derivatization process is described in paragraph 2.7.2 and 2.7.3.

All analyses were performed, using Waters ACQUITY UPLC® Photodiode Array (PDA) eλ Detector, connected with MassLynx™ Software (Waters Corporation, Milford, MA USA). Furthermore, an Acquity UPLC BEH (bridged ethylsiloxane/silica hybrid structure) C₁₈ column (2.1 mm × 150 mm, 1.7 μm) as stationary phase, also from Waters, was used. The column temperature was maintained at 43 °C. The system was checked by use of standard solutions with known amounts of the analytes. The standards and samples were separated using a mobile phase consisting of AccQ-Tag Ultra Eluent A - 1:10 dilution and AccQ-Tag Ultra Eluent B. The flow rate was set at 400 μL/min and the injection volume at 1 μL. The detection wavelength was set at 260 nm (see Appendix II).

The Waters AccQ-Tag Ultra Derivatization Kit was used for the UPLC amino acid analysis. Each kit of reagents consists of one vial each of:

- Ultra Borate Buffer – The buffer is added to the samples to ensure the optimum pH for derivatization.
- Ultra Reagent Powder – 6-aminoquinolyl-N-hydroxysuccinimidyl carbonate (AQC) derivatizing reagent.
- Ultra Reagent Diluent – This diluent, acetonitrile, is used to reconstitute the reagent for derivatization.

2.7.2 Sample preparation

The amino acid derivatives were prepared by mixing an aliquot of a sample (or of the standard solution containing 16 standard amino acids, Amino Acid Hydrolysate Standard, 10 x 1 mL WAT088122, Waters Corporation, Milford, MA USA) with the borate buffer and the derivatizing solution. The derivatizing solution was prepared according to instructions (Instruction manual, Appendix III), by dissolving the Ultra Reagent Powder in 1 ml Ultra Reagent Diluent. Furthermore, the solution was mixed for 10 seconds and heated on top of a heating block at 55°C (Stuart SBH200D/3 Stone, UK) for 15 minutes. During these 15 minutes, every five minutes the solution was vortexed (Vortex MS 3 basic Staufen Germany). After the reagent was reconstituted (see instruction manual, Appendix III), 10 μl of the sample was added and mixed for 15 seconds with 70 μl of Ultra Borate Buffer. Subsequently 20 μl of reconstituted AccQ•Tag™ Reagent was added to the vial and mixed for 10 seconds. After leaving it for one minute at room temperature the vial was heated up again for 10 minutes at 55 °C on the top of the heating block.

To avoid contamination of the sample, the preparation was performed inside a ventilated hood, wearing nitrile gloves.

Micropipettes (Proline Plus Pipette 0.5-10 μ l, 10-100 μ l, 20-200 μ l Sartorius Corporate Administration Gmb Weender Landstr., Goettingen) and Pipette tips (5-200 μ l Thermo Fisher Scientific Oy Vantaa, Finland) were used during the sample preparation.

2.7.3 Reaction scheme

The AccQ•Tag™ derivatization reagent reacts rapidly with amines through nucleophilic attack. The result is the loss of N-hydroxysuccinimide (NHS) and CO₂ (fig. 2.7.3). The resulting stable derivatives are now ready for analysis. Primary and secondary amino acids are derivatized quickly, on a milliseconds time scale, and they are stable for more than one week at room temperature (Instruction manual, Appendix III). The excess reagent reacts more slowly with water, hydrolyzing in a few tens of seconds (<http://www.knauer.net/>).

Problems were identified initially due to the use of Ortho-phthalaldehyde (OPA) (a different derivatization reagent for amino acids). OPA derivatization was excluded, as some amino acid derivatives were stable only for a few minutes.

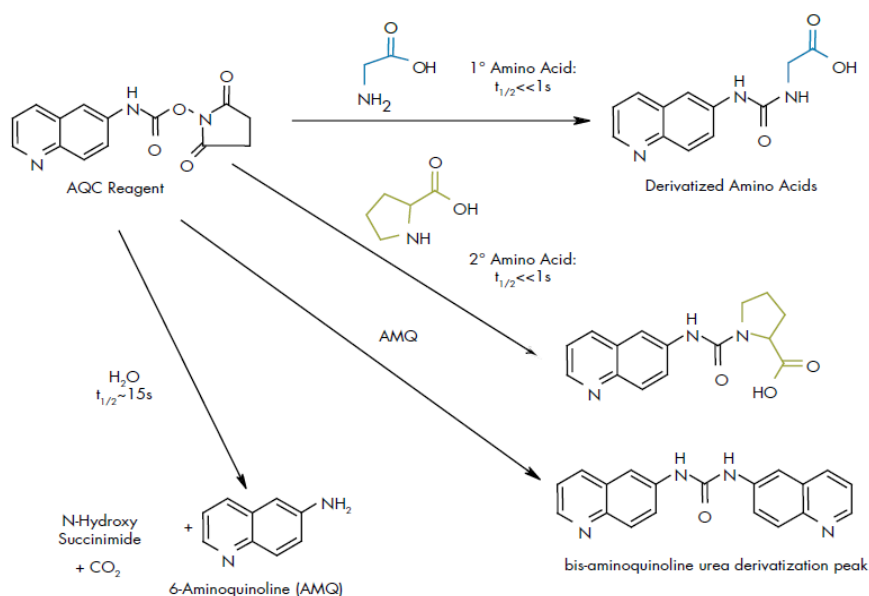


Figure 2.7.3 - Reaction scheme of AQC with primary and secondary amino acids (<http://www.waters.com/>)

2.7.4 Use of an internal standard

Internal standards (IS hereafter) are commonly used in chromatography. The D-Norvaline (Sigma-Aldrich Co. LLC St. Louis, Missouri), a structural isomer of Valine, was chosen as IS. This amino acid has a similar retention time and similar derivatization reaction as our analytes. This standard is stable, does not interfere with the sample and is not present in normal biological samples.

The correct concentration of each analyte was derived based on comparing their chromatogram peaks with those obtained by adding a known amount of Norvaline (to yield 100 pmol/ μ l), by integrating the area under the peaks formed for each analyte (including D-Norvaline). This was done also to correct the eventual slight variation of the instrument detector response.

2.7.5 Cleaning system

To ensure the quality of results the ACQUITY UPLC system was cleaned following the Waters MassTrak Amino Acid Analysis Solution System Guide, 2008 (see Appendix IV). Initially, failure to clean the system and to use solvents (e.g. acetonitrile and water) of HPLC grade, resulted in high background contamination, poor signal-to-noise ratio, and loss of sensitivity, with consequent difficulties in reading the chromatograms. This problem was corrected by using the correct type of cleaning procedure.

2.7.6 Calibration curves

A solution of mixed standards from Waters was prepared and diluted to the appropriate concentration for the establishment of calibration curves. Four concentration level standards (25, 50, 100 and 200 Pmol/ μ l) were injected in triplicates. Then the calibration curves were constructed by plotting the peak area of the analyte (e.g. Glu) divided by the peak area of the IS, versus the concentration of the standard analyte (e.g. Glu) divided the concentration of the IS (see Appendix V).

The IS was used in the formula below to correct the eventual slight variation of the instrument detector response and to extrapolate the real concentration of the analyte in the sample.

$$\frac{A_{analyte}}{A_{IS}} = m \frac{C_{analyte}}{C_{IS}} + q$$

The quotient Area Analyte/Area IS, is given by the peak area of the analyte (chromatogram peak) divided by the peak area of the IS. The quotient Concentration Analyte/Concentration IS, which take into account the final concentration in the solution, is given by the concentration of the analyte in sample (unknowns) divided by the concentration of the IS (100 pmol/ μ l, fixed concentration added to each sample). The slope m and y-intercept q are given by the calibration curves (obtained by use of the Waters standard) for each single analyte (Appendix V).

2.8 Statistics

The statistical analysis of data containing repeated measurement, allocated to different treatments over time, as in this case, may be challenging. Repeated measures data are often analyzed by repeated ANOVA. However, this model present limitations when there are missing measurements, or when some of the data are measured at different times. Such data are excluded resulting in insufficient information for a meaningful analysis. The presence of unequally (temporally) spaced and/or missing data does not pose a problem for the Mixed Model approach (Wang and L. A. Goonewardene, 2004). The Mixed Model uses the generalized least squares method, which is more powerful than the ordinary least squares used by a General Linear Model. Another key reason to use the Mixed Model was to analyse repeated measurement, something which is not possible with the General Linear Model.

Results of analyses of individual samples were obtained as the means of three replicate analyses, both for the controls and the treatments. Linear Mixed Model with Bonferroni correction for multiple measurements was used to determine significant differences between treatment and control and for pairwise comparison. Temporal efflux of neurotransmitter was also evaluated using the Mixed Model to identify the trend of the analyte over time.

In the data analysis, only repeated measures ($n=3$) with a CV below 10% were taken into consideration. All statistical tests were evaluated at the 95% confidence level as the threshold for significance. All the statistics is showed in Appendix VI.

Statistical analyses were carried out with IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.

3. Results

3.1 Chromatograms of Waters standard amino acids.

The standard chromatogram (figure 3.1.1) shows the separation of 16 standard Waters amino acids and the added Norvaline.

The reproducibility of the assay was shown by performing intra and inter-run precision tests. The intra-run precision test (n=3) was performed over a one day period (three vials with derivatized standard amino acids were analysed in triplicate at different time over a day) (table 3.1.1) and the inter-run precision (n=3) over a three weeks period (three vials with derivatized standard amino acids were analysed in triplicate at different time over a three weeks) (table 3.1.2). The %CV results for all amino acids of the intra-run and the inter-run tests were <2.6% and <3.6% respectively.

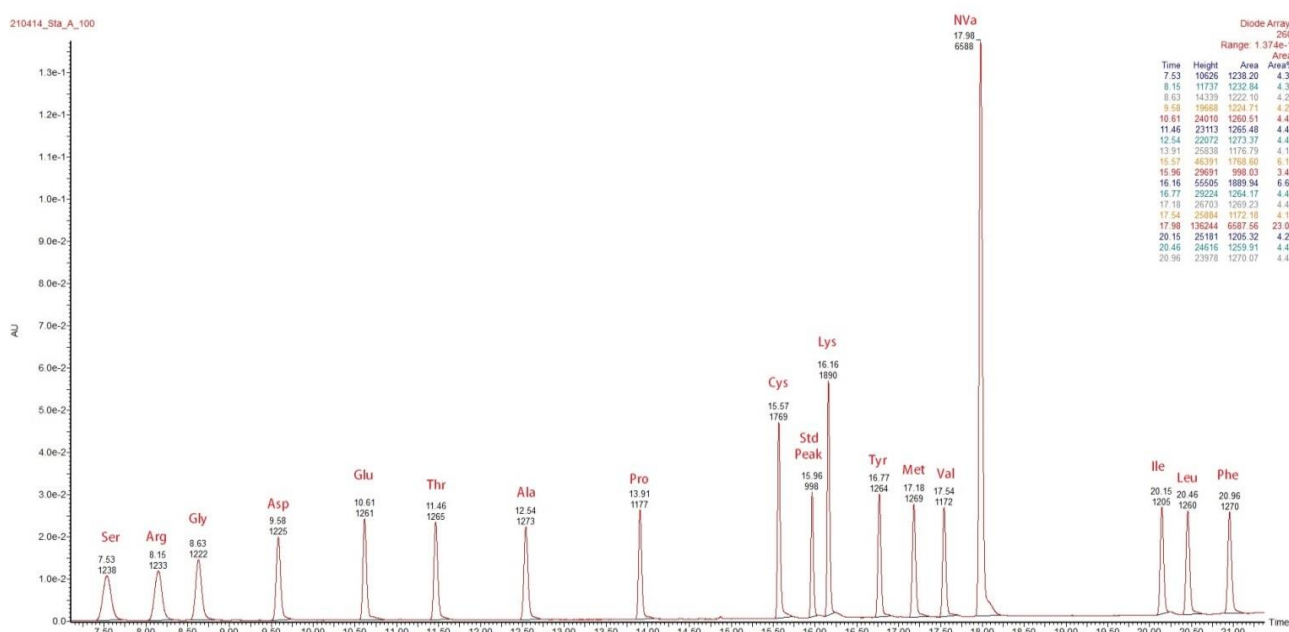


Figure 3.1.1 – Typical chromatogram showing the separation of Waters standard amino acids (100 Pmol/μL) using the UPLC amino acid analysis. The standard contains 16 amino acids and the Norvaline (NVA) (added afterwards) as the internal standard. X-axis: retention time. Y-axis: absorbance (AU). Std Peak: Derivatization reagent peak. The first number above the peak indicate the exact retention time (X-axis). The second number above the peak indicate the peak area.

Amino acid	Peak area (100 pmol/μL)	SD (100 Pmol/μL)	CV (%)
Glu	1153	29.9	2.6
Ser	1236	14.9	1.2
Gly	1224	6.8	0.5
Thr	1253	5.5	0.4

Table 3.1.1 - Intra-run precision test performed over one day with the Waters standard amino acids of interest (100 pmol/μL). Peak area: Mean peak area as derived from three intra-run tests, each with triplicate analyses, SD: standard deviation, CV: coefficient of variation.

Amino acid	Peak area (100 Pmol/μL)	SD (100 Pmol/μL)	CV (%)
Glu	1172	42.6	3.6
Ser	1244	10.9	0.9
Gly	1228	13.6	1.1
Thr	1246	23.0	1.8

Table 3.1.2 - Inter-run precision test performed over a three weeks period with the Waters standard amino acids of interest (100 pmol/μL). Peak area: Mean peak area as derived from three inter-run tests, each with triplicate analyses, SD: standard deviation, CV: coefficient of variation.

Standard chromatograms comparison showed that there was no difference in the retention time between Waters standard amino acids diluted in water and in aCSF (figure 3.1.2 – 3.1.3). According to this, looking at the retention time, it was assumed that unknown peaks in aCSF samples were represented by the amino acid that had the same/similar retention time in the Waters standard.

Blank samples containing only water and blank samples containing only the matrix (aCSF), were derivatized. The result was a single visible peak (the derivatization peak), without cross-reactions between the derivatization agent and components of the aCSF. Contaminants are not present within the matrix and the Millipore water (figure 3.1.4 – 3.1.5).

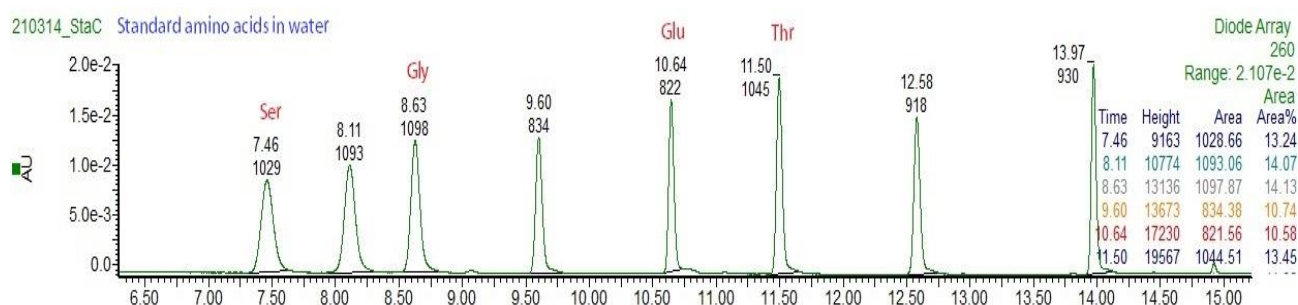


Figure 3.1.2 – Chromatogram showing the separation of Waters standard amino acids diluted in water (100 Pmol/μL). X-axis: retention time. Y-axis: absorbance (AU). The first number above the peak indicates the exact retention time (X-axis). The second number above the peak indicates the peak area.

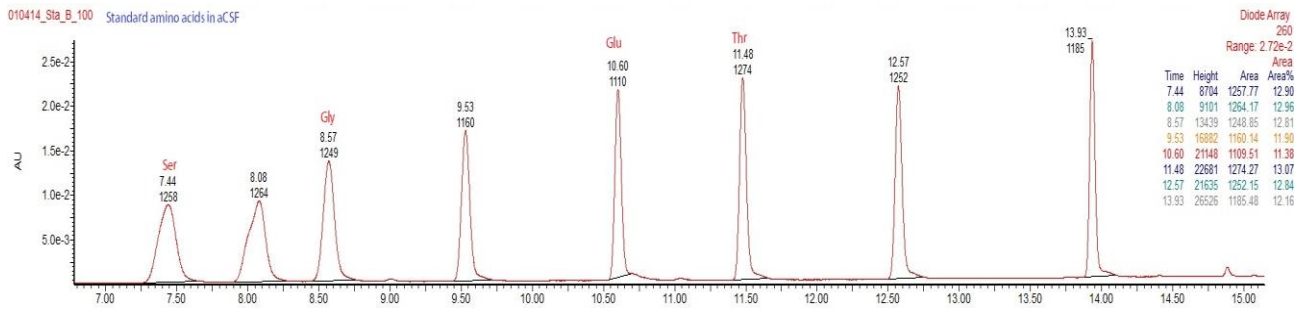


Figure 3.1.3 – Chromatogram showing the separation of Waters standard amino acids diluted in aCSF (100 Pmol/μL). X-axis: retention time. Y-axis: absorbance (AU). The first number above the peak indicates the exact retention time (X-axis). The second number above the peak indicates the peak area.

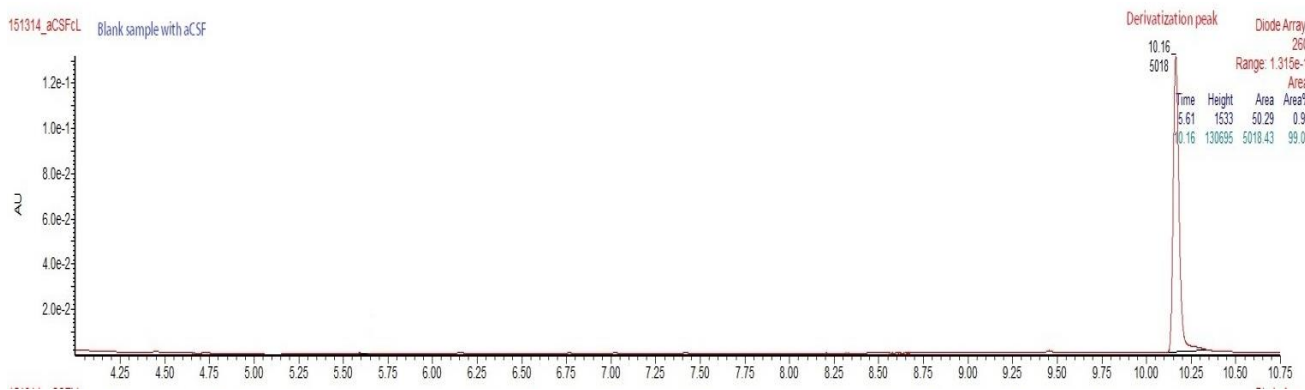


Figure 3.1.4 – Chromatogram showing the derivatized blank matrix (aCSF) sample. X-axis: retention time. Y-axis: absorbance (AU). The first number above the peak indicates the exact retention time (X-axis). The second number above the peak indicates the peak area.

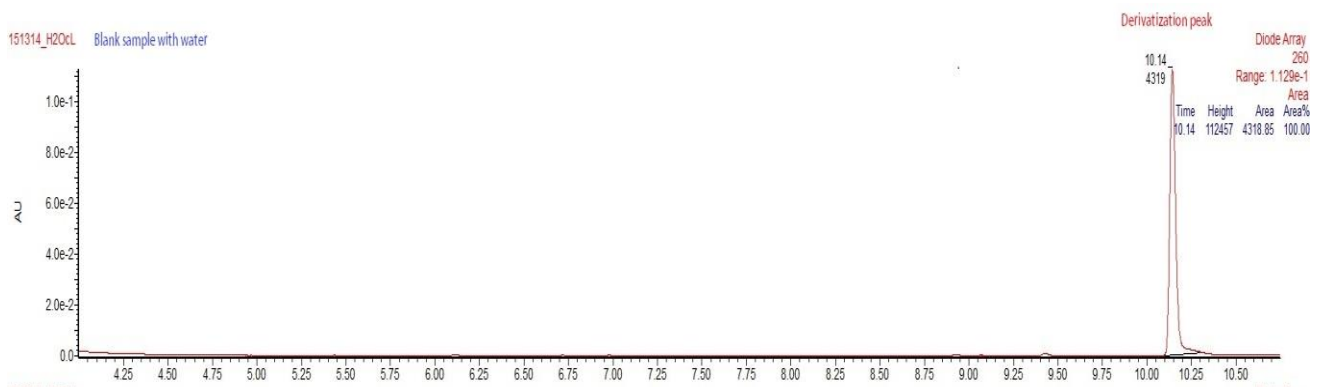


Figure 3.1.5 – Chromatogram showing the derivatized blank Millipore water sample. X-axis: retention time. Y-axis: absorbance (AU). The first number above the peak indicates the exact retention time (X-axis). The second number above the peak indicates the peak area.

3.2 Chromatograms of duck and chicken samples

Ultra Performance Liquid Chromatography (UPLC), on physiological samples of eider duck and chicken, showed good peak resolution and low chromatographic interferences (figure 3.2.1, 3.2.2, 3.2.3).

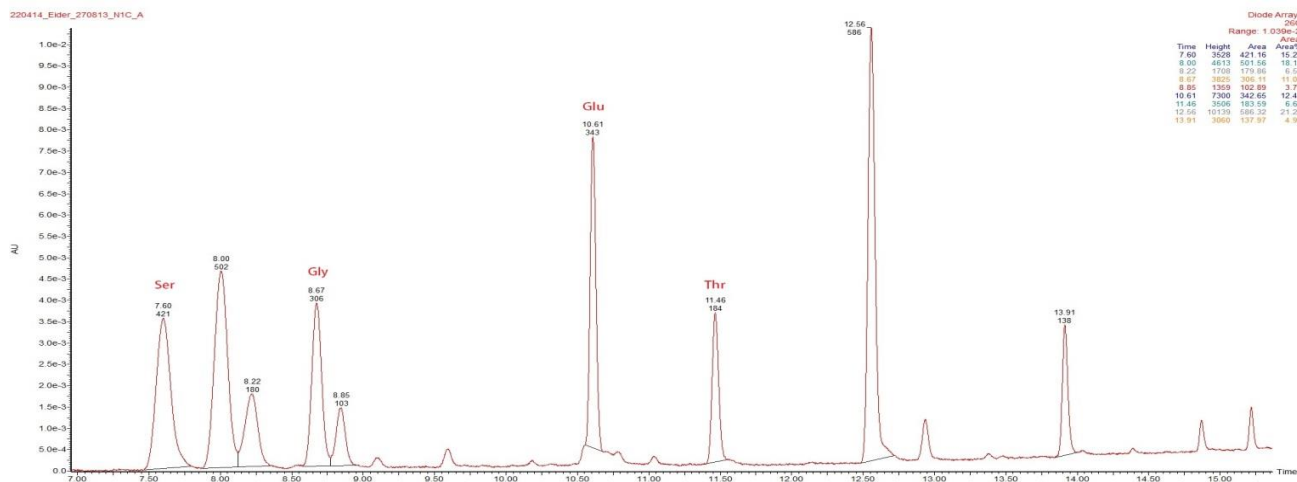


Figure 3.2.1 – Typical example of chromatogram showing derivatized physiological sample of eider duck, during the first three minutes sampling in normoxia exposure. X-axis: retention time. Y-axis: absorbance (AU). The first number above the peak indicate the exact retention time (X-axis). The second number above the peak indicate the peak area.

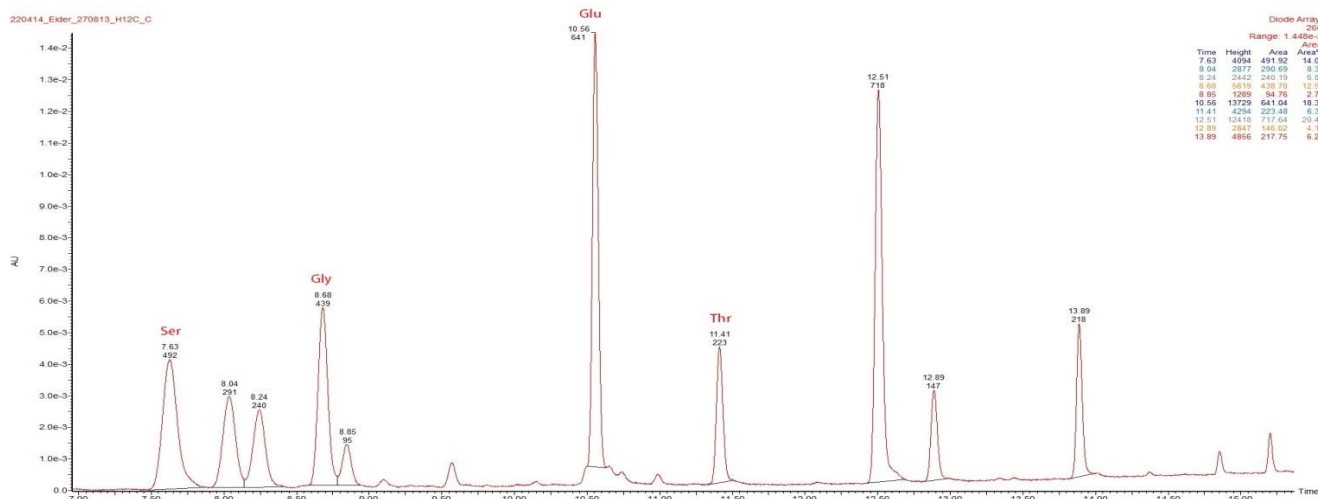


Figure 3.2.2 – Typical example of chromatogram showing derivatized physiological sample of eider duck, during hypoxia exposure (minute 39). X-axis: retention time. Y-axis: absorbance (AU). The first number above the peak indicate the exact retention time (X-axis). The second number above the peak indicate the peak area.

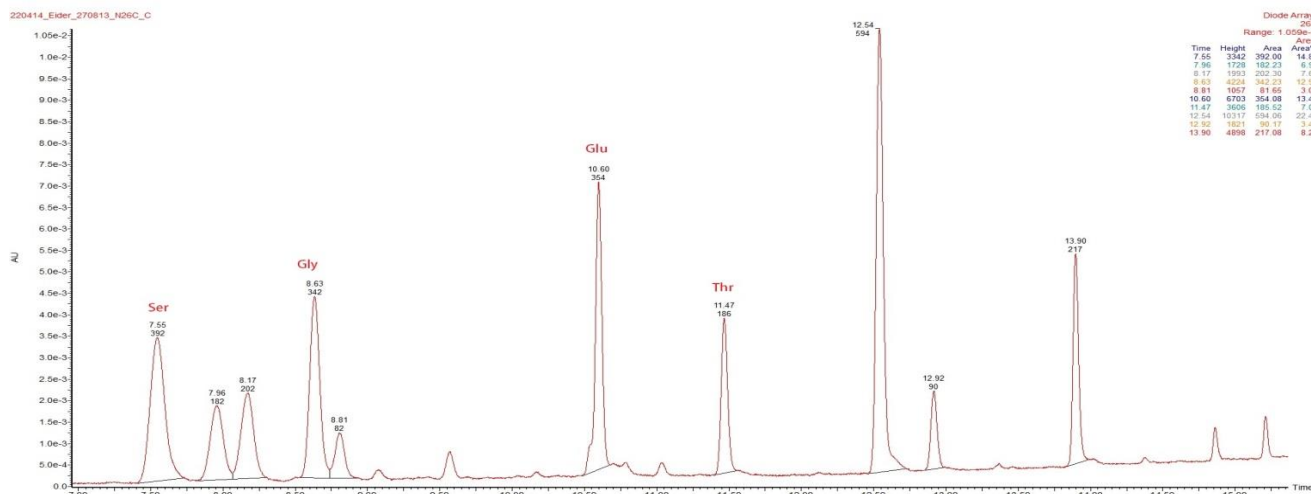


Figure 3.2.3 – Typical example of chromatogram showing derivatized physiological sample of eider duck, during recovery in normoxia, after hypoxia exposure (minute 84). X-axis: retention time. Y-axis: absorbance (AU). The first number above the peak indicate the exact retention time (X-axis). The second number above the peak indicate the peak area.

3.3 Efflux of neurotransmitter.

Cerebellum slices were cut following the procedure explained in the paragraph 2.5. The success of the procedure was proven by electrophysiological population recordings, showing that slices were still viable after the treatment (Appendix VII).

Analysis were performed to monitor the efflux of Glu, Ser, Thr and Gly in the perfusion experiments.

The standard curve (see paragraph 2.7.5 and Appendix V) was used to calculate the actual concentration of each peak area given by the chromatogram. The regression lines for concentrations versus peak areas for Gly, Ser, Thr and Glu showed coefficients (R^2) ranging from 0.9994 - 1.00 (see Appendix V).

Concentration calculations.

Results are means of three replicates. The mean peak area was adjusted using the Internal Standard, Norvaline, according to the formula presented in paragraph 2.7.5.

$$\frac{A_{\text{analyte}}}{A_{IS}} = m \frac{C_{\text{analyte}}}{C_{IS}} + q$$

In order to adjust the amounts of released amino acids for differences in the size of slices used, the amino acid values were divided by the weight of each brain slice (wet weight). The final value is expressed is Pmol/ μ l per gram tissue.

Cerebellum slice weight

At the end of the microperfusion experiments, each cerebellum slice was removed from the microperfusion chamber and weighted. The result are expressed in grams wet weight in table 3.3.

ID	Treatment eider duck (g) wet weight	Control eider duck (g) wet weight	Treatment Chicken (g) wet weight	Control Chicken (g) wet weight
160913			0.0643	0.0699
180913			0.0537	0.0560
200913			0.0458	0.0479
230913			0.0861	0.0647
270813	0.1055	0.1083		
290813	0.1048	0.0597		
020913	0.0726	0.0734		
060913	0.0752	0.0786		
Average	0.0895	0.0800	0.0625	0.0596

Table 3.3 – Cerebellum slice weight for chickens and eider ducks. ID: The animal ID correspond to the date of the experiment, already showed in table 2.1.1 and table 2.1.2.

Results

A Linear Mixed Model with Bonferroni correction for multiple measurements was used to determine significant differences between treatment and control and for pairwise comparison. The results were evaluated at the 95% confidence level as threshold for significance.

Upon hypoxia exposure, Glu release from eider duck cerebellar slices (fig. 3.4.1) showed a rise (minute 27), followed by a slow decrease which continued into the reoxygenation phase. Normoxic control slices showed no increase, only a slow decrease throughout the perfusion experiment. Glu release from chicken cerebellar slices (fig. 3.4.2) showed a slightly increased, not confirmed by pairwise comparison, followed by a decrease into the reoxygenation phase. Normoxic control slices showed quite stable efflux of Glu throughout the perfusion experiment.

Thr (fig. 3.4.3) and Ser (fig. 3.4.5) and Gly (fig. 3.4.7) release from eider duck cerebellar slices showed a rise, after minute 27, consistent throughout both the hypoxia and the reoxygenation phase. Normoxic control slices showed quite stable efflux throughout the perfusion experiment. Efflux from chicken cerebellar slices (fig. 3.4.4, 3.4.6 and 3.4.8) showed similar trend for Thr, Ser

and Gly but not confirmed by pairwise comparison. Normoxic control slices showed a slow decrease of Ser efflux throughout the perfusion experiment, also confirmed by pairwise comparison.

There was a significant difference between the treatment (hypoxia) and the control (normoxia) for Glu, Thr, Ser and Gly in both eider ducks and chickens ($p < 0,05$), with a higher levels of neurotransmitter efflux during the hypoxia exposure (tab. 3.4.1) (fig. 3.4.1 to fig. 3.4.8). Temporal variation was a significant factor only for the efflux of Glu in the eider ducks group ($F: 3,300_{5,41} p = 0,014$) (tab. 3.4.2) and for the efflux of Ser in the chickens group ($F: 2,699_{5,41} p = 0,034$). There was no significant change with time in the efflux of the different amino acids, in different experiments (tab.3.4.3).

Pairwise comparisons showed that the efflux of all amino acids (fig. 3.4.1, 3.4.3, 3.4.5, 3.4.7 and tab 3.4.4) was significantly higher for hypoxia-exposed eider duck slices than for controls. The response to hypoxia exposure seems to be more relevant in eider ducks rather than in chickens.

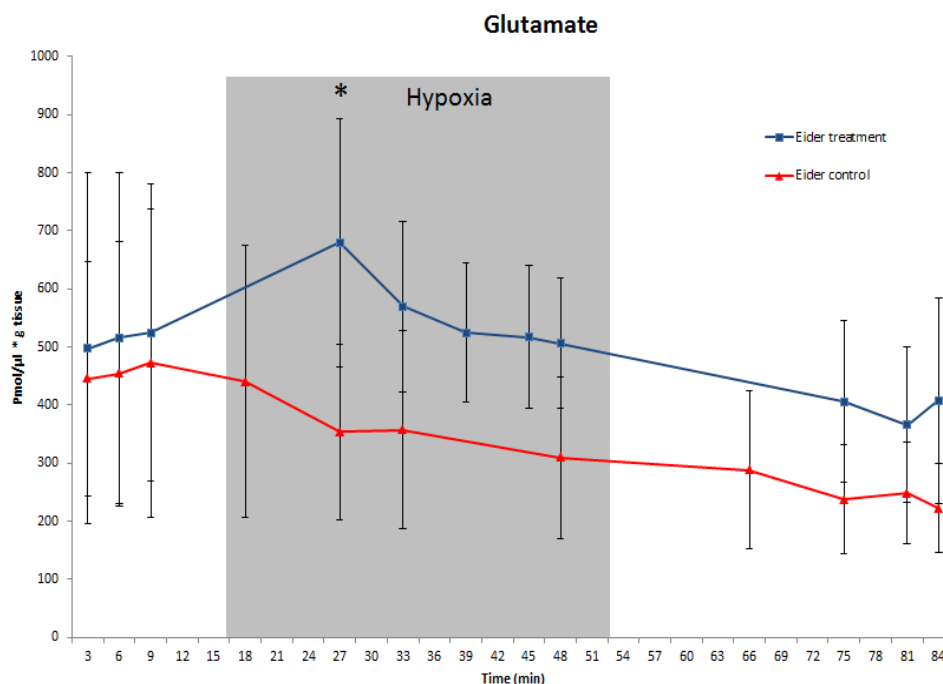


Figure 3.4.1 – Efflux of Glutamate over time in four eider ducks. Blue: treatment (hypoxia followed by reoxygenation), red: control (normoxia throughout). Shaded (grey area) period of hypoxia exposure for treatment slices. Data are given as means \pm standard deviation between individuals. Asterisk marks pair-wise significant difference ($p < 0.05$) between treatment and control (based on the linearly independent pairwise comparisons among the estimated marginal means).

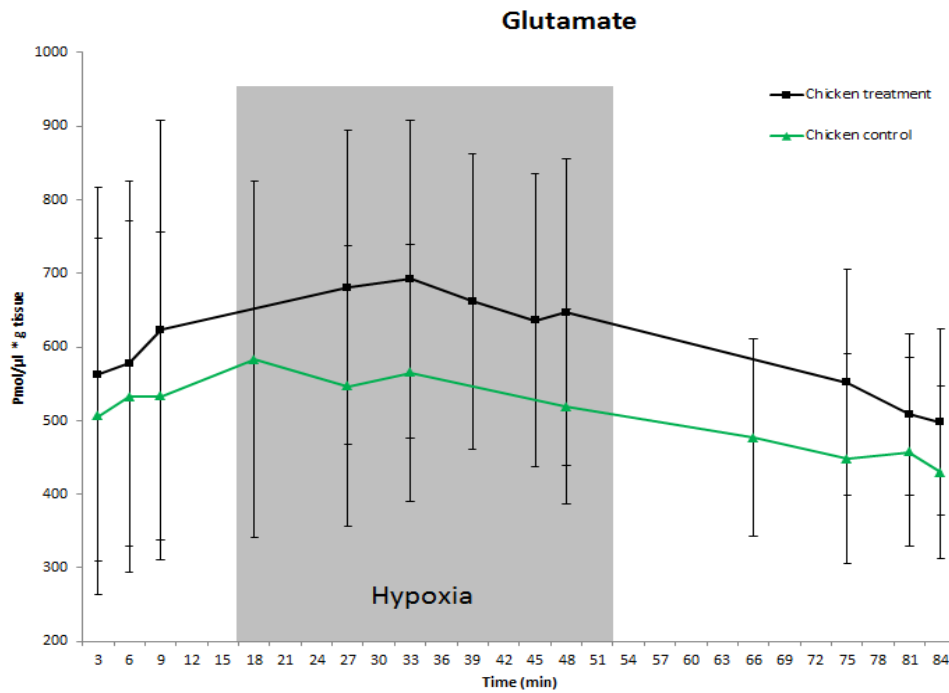


Figure 3.4.2 – Efflux of Glutamate over time in four chickens. Black: treatment (hypoxia followed by reoxygenation), green: control (normoxia throughout). Shaded (grey area) period of hypoxia exposure for treatment slices. Data are given as means \pm standard deviation between individuals.

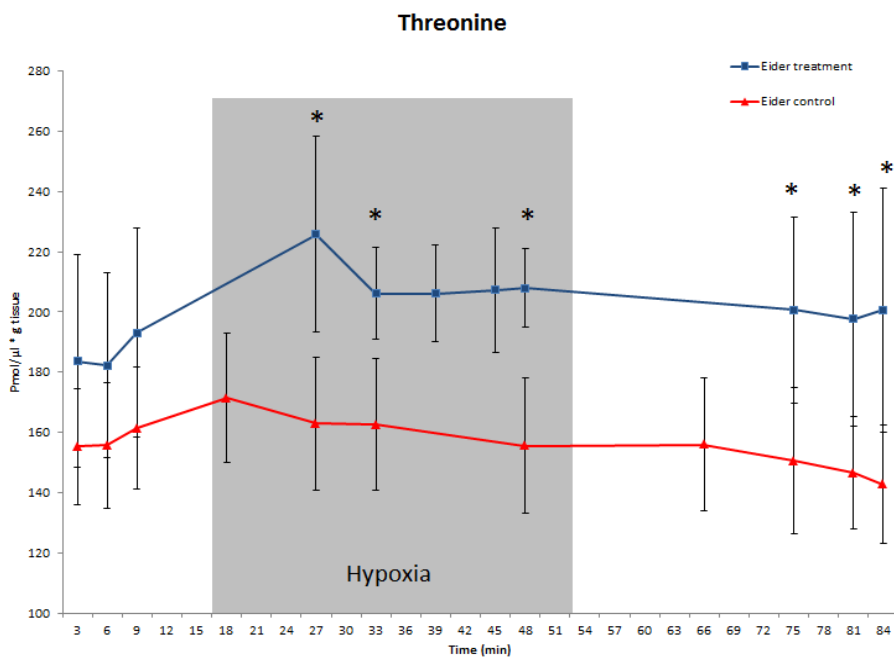


Figure 3.4.3 – Efflux of Threonine over time in four eider ducks. Blue: treatment (hypoxia followed by reoxygenation), red: control (normoxia throughout). Shaded (grey area) period of hypoxia exposure for treatment slices. Data are given as means \pm standard deviation between individuals. Asterisk marks pair-wise significant difference ($p < 0.05$) between treatment and control (based on the linearly independent pairwise comparisons among the estimated marginal means).

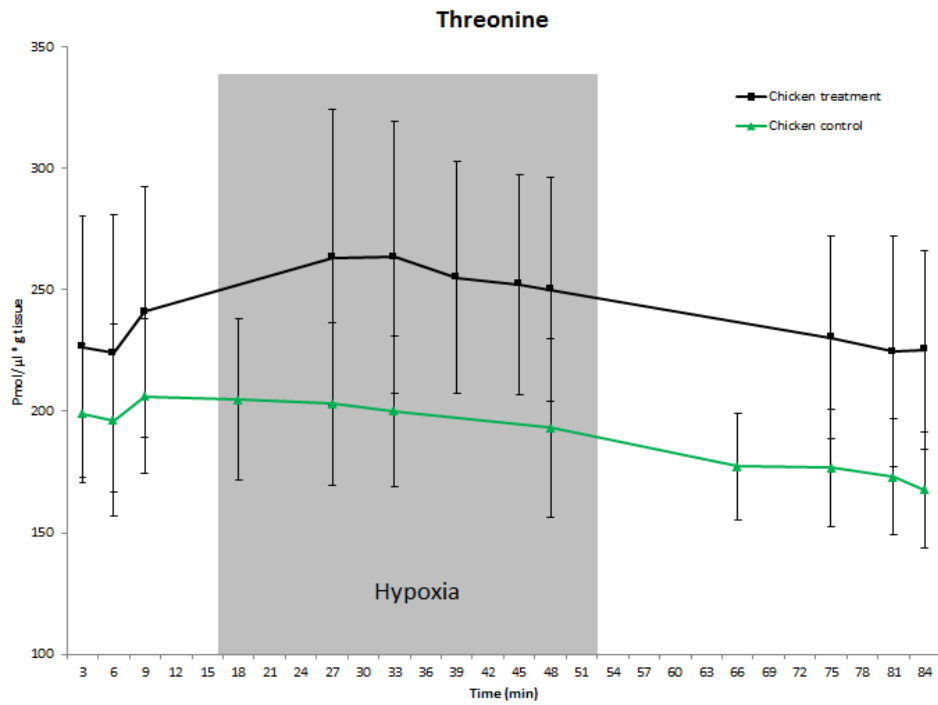


Figure 3.4.4 – Efflux of Threonine over time in four chickens. Black: treatment (hypoxia followed by reoxygenation), green: control (normoxia throughout). Shaded (grey area) period of hypoxia exposure for treatment slices. Data are given as means \pm standard deviation between individuals.

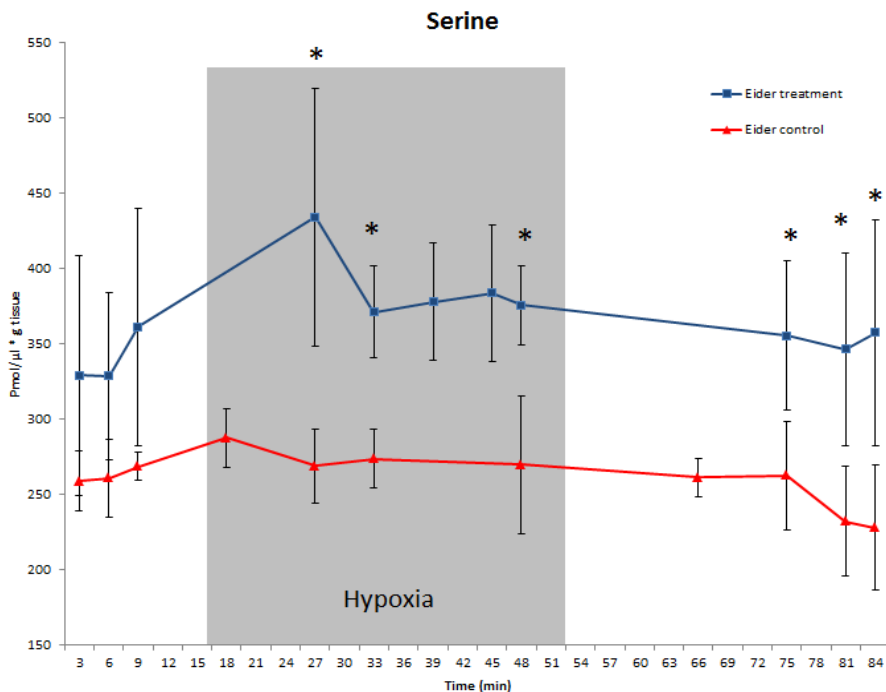


Figure 3.4.5 – Efflux of Serine over time in four eider ducks. Blue: treatment (hypoxia followed by reoxygenation), Red: control (normoxia throughout). Shaded (grey area) period of hypoxia exposure for treatment slices. Data are given as means \pm standard deviation between individuals. Asterisk marks pair-wise significant difference ($p < 0.05$) between treatment and control (based on the linearly independent pairwise comparisons among the estimated marginal means)

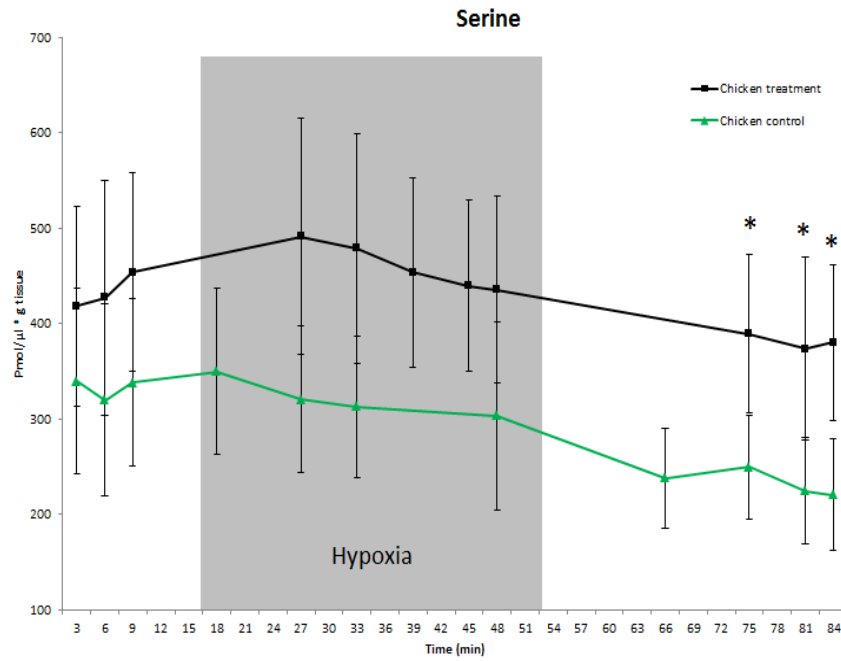


Figure 3.4.6 – Efflux of Serine over time in four chickens. Black: treatment (hypoxia followed by reoxygenation), green: control (normoxia throughout). Shaded (grey area) period of hypoxia exposure for treatment slices. Data are given as means \pm standard deviation between individuals. Asterisk marks pair-wise significant difference ($p < 0.05$) between treatment and control (based on the linearly independent pairwise comparisons among the estimated marginal means)

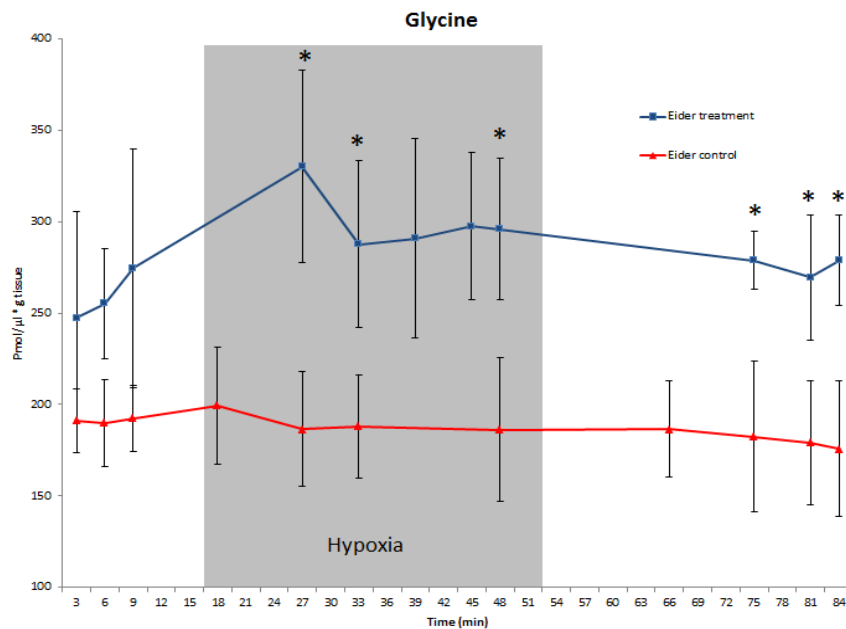


Figure 3.4.7 – Efflux of Glycine over time in four eider duck. Blue: treatment (hypoxia followed by reoxygenation), Red: control (normoxia throughout). Shaded (grey area) period of hypoxia exposure for treatment slices. Data are given as means \pm standard deviation between individuals. Asterisk marks pair-wise significant difference ($p < 0.05$) between treatment and control (based on the linearly independent pairwise comparisons among the estimated marginal means).

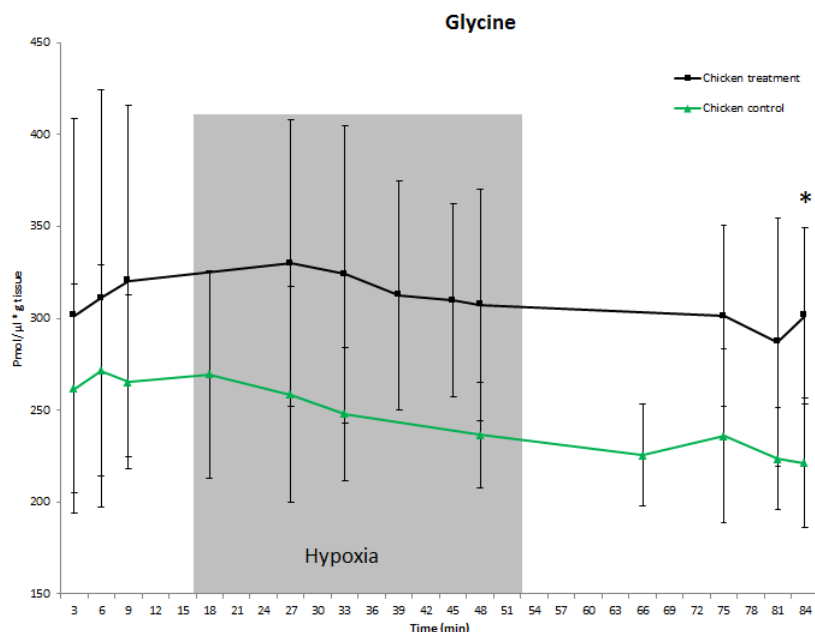


Figure 3.4.8 – Efflux of Glycine over time in four chickens. Black: treatment (hypoxia followed by reoxygenation), green: control (normoxia throughout). Shaded (grey area) period of hypoxia exposure for treatment slices. Data are given as means \pm standard deviation between individuals. Asterisk marks pair-wise significant difference ($p < 0.05$) between treatment and control (based on the linearly independent pairwise comparisons among the estimated marginal means).

Effect of the experiment (treatment and control) - Test of Fixed Effect¹

Chemicals	Species	Sig. ^a
Glutamate	Eider	.000
Glutamate	Chicken	.032
Threonine	Eider	.000
Threonine	Chicken	.000
Serine	Eider	.000
Serine	Chicken	.000
Glycine	Eider	.000
Glycine	Chicken	.000

Table 3.4.1 – Significant difference between the treatment (hypoxia) and the control (normoxia) for Glu, Thr, Ser and Gly in both eider ducks and chickens. 1: Looking at the effect of the experiment (treatment and control). Fixed factors: Experiment (treatment and control) a: Adjustment for multiple comparisons: Bonferroni. Data were split for chemical and species.

Effect of the temporal variation (time) on the experiment (treatment and control) -
Test of Fixed Effect¹

Chemicals	Species	Sig. ^a
Glutamate	Eider	.014
Glutamate	Chicken	.151
Threonine	Eider	.425
Threonine	Chicken	.203
Serine	Eider	.122
Serine	Chicken	.034
Glycine	Eider	.442
Glycine	Chicken	.657

Table 3.4.2 - Significant difference between the treatment (hypoxia) and the control (normoxia) for Glu, Thr, Ser and Gly in both eider ducks and chickens. 1: Looking at the effect of the temporal variation (time) on the experiment (treatment and control). Fixed factors: Experiment (treatment and control), time. a: Adjustment for multiple comparisons: Bonferroni. Data were split for chemical and species.

Assumption of different slopes - Test of Fixed Effect¹

Chemicals	Species	Sig. ^a
Glutamate	Eider	.791
Glutamate	Chicken	.993
Threonine	Eider	.984
Threonine	Chicken	.000
Serine	Eider	.698
Serine	Chicken	.997
Glycine	Eider	.740
Glycine	Chicken	1.000

Table 3.4.3 - Significant difference between the treatment (hypoxia) and the control (normoxia) for Glu, Thr, Ser and Gly in both eider ducks and chickens. 1: Assumption of different slopes. Fixed factors: Experiment (treatment and control), time, interaction time and experiment. a: Adjustment for multiple comparisons: Bonferroni. Data were split for chemical and species.

Pair-wise significant difference

Chemicals	Species	Time (min)	Sig. ^a
Glutamate	Eider	27	.047
Threonine	Eider	27	.019
Threonine	Eider	33	.017
Threonine	Eider	48	.007
Threonine	Eider	75	.044
Threonine	Eider	81	.043
Threonine	Eider	84	.042
Serine	Eider	27	.010
Serine	Eider	33	.002
Serine	Eider	48	.007
Serine	Eider	75	.023
Serine	Chicken	75	.031
Serine	Eider	81	.021
Serine	Chicken	81	.036
Serine	Eider	84	.023
Serine	Chicken	84	.019
Glycine	Eider	27	.003
Glycine	Eider	33	.010
Glycine	Eider	48	.007
Glycine	Eider	75	.005
Glycine	Eider	81	.010
Glycine	Eider	84	.004
Glycine	Chicken	84	.036

Table 3.4.4 - Pair-wise significant difference ($p < 0.05$) between treatment (hypoxia) and control (normoxia) (based on the linearly independent pairwise comparisons among the estimated marginal means for Glu, Thr, Ser and Gly in both eider ducks and chickens. Fixed factors: Time. a: Adjustment for multiple comparisons: Bonferroni. Data were split for chemical, time and species.

There was a significant difference in the effect of treatment, between the eider duck and chicken group for Glu (F: 5,439_{1,41} $p = 0,025$), Thr (F: 24,107_{1,86} $p < 0,001$), Ser (F: 14,246_{1,86} $p < 0,001$), and Gly (F: 4,770_{1,85} $p = 0,032$) (tab. 3.4.5). Higher levels of neurotransmitter efflux during the hypoxia, and normoxia exposure were present in the chicken group (fig. 3.4.9 to fig. 3.4.12). Temporal variation was a significant factor only for the efflux of Glu (F: 3,230_{5,41} $p = 0,015$) (tab. 3.4.6).

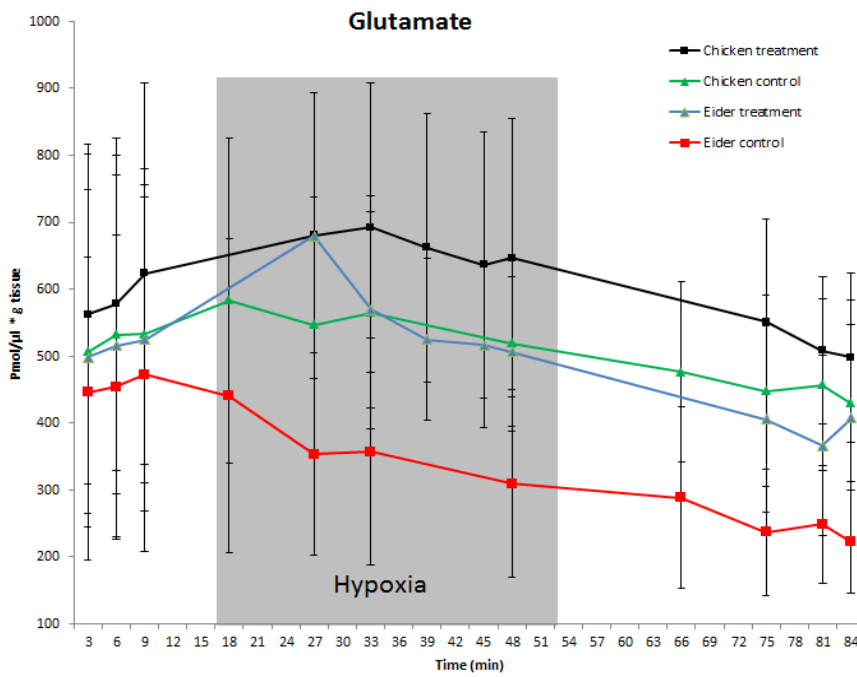


Figure 3.4.9 – Efflux of Glutamate over time in four eider duck and four chickens. Black: treatment (hypoxia followed by reoxygenation) in chickens, blue: treatment (hypoxia followed by reoxygenation) in eider duck. Red: control (normoxia throughout) in eider duck. Green: control (normoxia throughout) in chicken. Shaded (grey area) period of hypoxia exposure for treatment slices. Data are given as means \pm standard deviation between individuals.

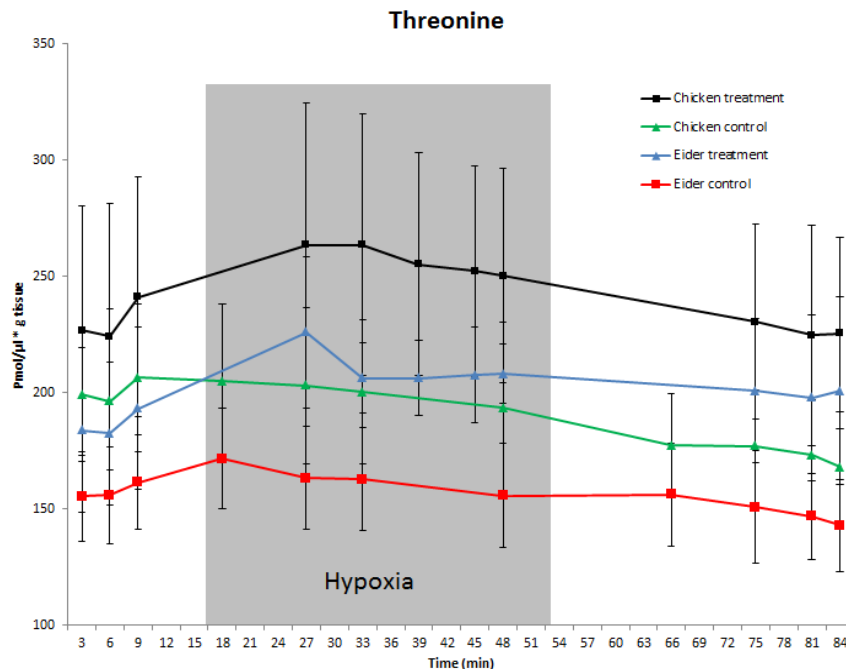


Figure 3.4.10 – Efflux of Threonine over time in four eider duck and four chickens. Black: treatment (hypoxia followed by reoxygenation) in chickens, blue: treatment (hypoxia followed by reoxygenation) in eider duck. Red: control (normoxia throughout) in eider duck. Green: control (normoxia throughout) in chicken. Shaded (grey area) period of hypoxia exposure for treatment slices. Data are given as means \pm standard deviation between individuals.

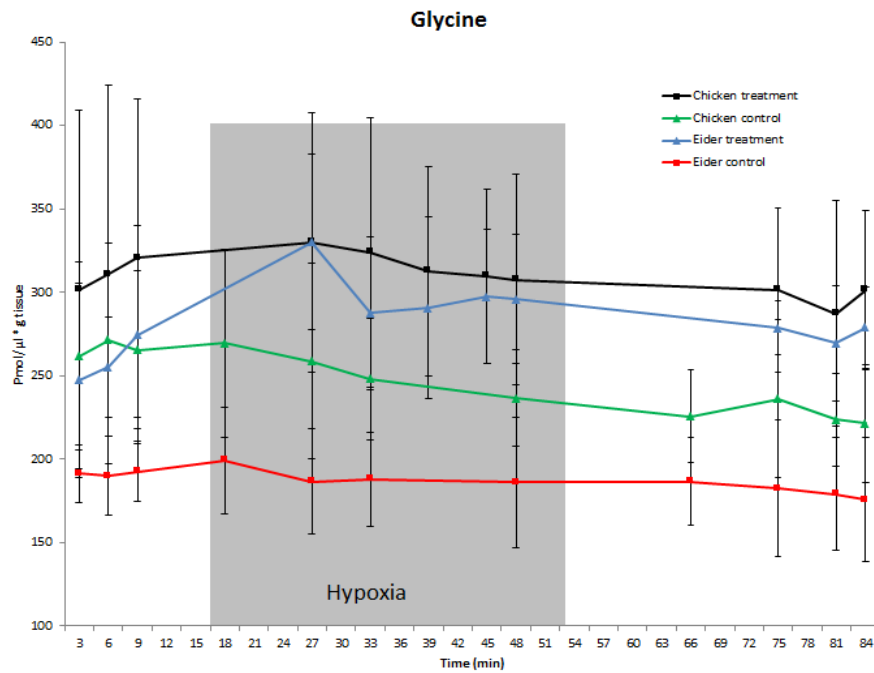


Figure 3.4.11 – Efflux of Glycine over time in four eider duck and four chickens. Black: treatment (hypoxia followed by reoxygenation) in chickens, blue: treatment (hypoxia followed by reoxygenation) in eider duck. Red: control (normoxia throughout) in eider duck. Green: control (normoxia throughout) in chicken. Shaded (grey area) period of hypoxia exposure for treatment slices. Data are given as means \pm standard deviation between individuals.

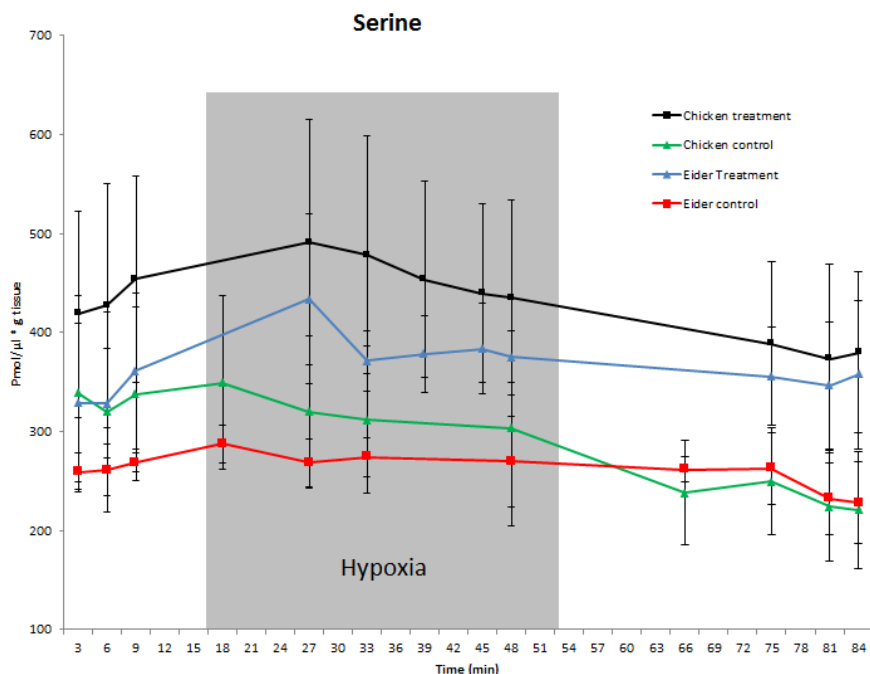


Figure 3.4.12 – Efflux of Serine over time in four eider duck and four chickens. Black: treatment (hypoxia followed by reoxygenation) in chickens, blue: treatment (hypoxia followed by reoxygenation) in eider duck. Red: control (normoxia throughout) in eider duck. Green: control (normoxia throughout) in chicken. Shaded (grey area) period of hypoxia exposure for treatment slices. Data are given as means \pm standard deviation between individuals

Significant difference in the effect of treatment and control, between the eider duck and chicken.
 Test of Fixed Effect¹

Chemicals	Experiment	Sig. ^a
Glutamate	Control	.000
Glutamate	Treatment	.025
Threonine	Control	.000
Threonine	Treatment	.000
Serine	Control	.021
Serine	Treatment	.000
Glycine	Control	.000
Glycine	Treatment	.032

Table 3.4.5 – Significant difference in the effect of treatment and control, between the eider duck and chicken for Glu, Thr, Ser and Gly. 1: Looking at the effect of the treatment and control. Fixed factor: Species. a: Adjustment for multiple comparisons: Bonferroni. Data were split for chemicals and experiment.

Effect of the temporal variation (time).
 Test of Fixed Effect¹

Chemicals	Experiment	Sig. ^a
Glutamate	Control	.175
Glutamate	Treatment	.015
Threonine	Control	.127
Threonine	Treatment	.439
Serine	Control	.029
Serine	Treatment	.099
Glycine	Control	.754
Glycine	Treatment	.426

Table 3.4.6 – Significant difference in the effect of treatment and control, between the eider duck and chicken for Glu, Thr, Ser and Gly. 1: Looking at the effect of the temporal variation (time). Fixed factors: Species and time. a: Adjustment for multiple comparisons: Bonferroni. Data were split for chemicals and experiment.

4. Discussion

4.1 Potential sources of errors

Use of Ultra Performance Liquid Chromatography (UPLC) to analyse the amino acid composition in physiological samples, showed good peak resolution and low chromatographic interferences (fig. 3.2.1, 3.2.2, 3.2.3), which suggests that the Waters MassTrak Amino Acid Analysis (AAA) Solution (Anonymous, 2008) was a reliable method for these analyses.

Electrophysiological population recordings (Appendix VII), showed clear slice vitality, with spontaneous discharge activity that is typical for the Purkinje layer of duck cerebellar slices (e.g., Ludvigsen and Folkow 2009). The same recording was performed on cerebellum slices after the microperfusion experiment. Vitality, even if weaker, showed that the slices survived after 84 minutes of microperfusion for both treatment and control.

The proven vitality of slices, indicates that the correct settings were chosen for perfused flow rate (0.0234 ± 0.0015 ml/min, see Appendix VII). The flow rate was adjusted by taking into consideration the average size of the slices in the study by Kirschner et al. (2009). Consequently, the perfusion flow rate for cerebellum slices was increased by three times compared to that used for hippocampus slice ($7 \mu\text{l}/\text{min}$) by Kirschner et al. (2009).

The Waters MassTrak™ method, for use in the high throughput analysis of amino acids, was found robust, reliable, and reproducible (see discussions 4.2)

4.2 Development of the method analysis

Early advice on use of the Ultra-Performance Liquid Chromatographic (UPLC) analysis of amino acids that proved to be wrong, led to some initial mistakes and problems with proper reading and interpretation of the results. In the beginning, chromatograms showed interfering peaks and baseline disturbances. These methodological hitches had their roots in the wrong use of the mobile phase and the fact that the need for a routine cleaning process of the instrument (see Appendix IV) was not considered. These routines were changed after a conference with the Waters company. Initially, self-made eluents (water/acetonitrile and acetonitrile/formic acid, commonly used for amino acid analysis) were used, but, again after consultation with the Waters company, their advice of using the Waters MassTrak™AAA kit was followed. This proved to be a robust, reliable, and reproducible method (see paragraph 4.3), and inaccuracies were considerably reduced (at a higher cost of the analysis) by using the complete original Waters kit and protocol

(Anonymous, 2008). In order to reduce the analysing time and cost, the running time was reduced from 45 minutes (Anonymous, 2008) to 32 minutes. The results, after run time shortening, still showed no critical peak interference (Appendix II).

4.3 Validation of the method

Linearity

The linearity of the method of the mathematical relationship between the different concentration of the analyte (pmol/ μ l) and the response peak area, graphically represented as a straight line, was calculated by analyzing standard amino acids from Water (fig. 3.1.1) at four different concentration levels (25, 50, 100 and 200 pmol/ μ l). The calibration curve was constructed by plotting the response area of the analyte divided by the response area of the Internal Standard (Norvaline), against the corresponding concentration injected of the analyte divided by the known concentration of injected Norvaline, using the least square method (Appendix V). The high value of the correlation coefficient (r^2) 0.999 for Glu, Thr, Ser and Gly calibration curve, indicates good linearity (Kumar et al., 2011).

Specificity

The capability of this method to separate and accurately measure the peaks of interest (fig. 3.1.1) indicates the specificity of the method. The specificity of the method was checked by injecting standard amino acids from Waters, together with the aCSF sample from the microperfusion experiment (fig. 3.1.2). No interference of the matrix components was observed, with regard to the retention time of analyte peak (Kumar et al., 2011, Rane et al., 2012).

Precision

The precision of the chromatographic method, was estimated by measuring repeatability and time-dependent intermediate precision based on three replicated injections. The low CV values (%) presented in tab. 3.1.1 and 3.1.2 illustrate the good precision of the analytical method. In the data analysis, only repeated measures (n=3) with a CV below 10% were taken into consideration.

Solution stability

The stability of amino acid in the aCSF and standard solution was tested. The samples and standard amino acids were stored at -20 °C, at the most for almost one year. All the samples were

injected into the UPLC system at different time, throughout 8 weeks. The chromatographs of the stored samples were reliable; standards showed high repeatability independent of age and no additional/anomalous peaks were registered.

4.4 Brain slice vitality

The brain-slice technique is utilized mainly in electrophysiology (Wang and Kass, 1997). In this study, electrophysiological recordings of neuronal population activities were only used to monitor the vitality of slices before and after microperfusion in aCSF. Recorded activity was taken to indicate that slices were still alive after the experimental treatment (see Appendix VII).

Obviously, the procedures described (paragraph 2.3-2.6) had the objective to keep the brain tissue alive. Some care must be taken in each step to avoid problems, which may be due to the following potential sources of errors:

- The process of decapitation and extraction of the brain, before the slice is placed in the aCSF bath solution, may have effects on the brain tissue. Less than 5 minute were used to extract the brain after decapitation. Extraction and brain manipulation were conducted in a cold aCSF bath (< 5°). Low aCSF temperature slows down the metabolic rate and allows cells to survive the ischemic period during carrying and dissecting.
- The slicing method may itself compromise the brain tissue. Slicing of the tissue block can damage the top and bottom of the section. During microperfusion, the tissue also "ages", degrading at a faster rate than in the intact animal brain. Last but not least, the aCSF composition requires the necessary compounds in the right concentration (Sveistrup, 2005, Anonymous, 2011).
- The most delicate step is the transfer of the cerebellum slice into the microperfusion chamber. The brain tissue was handled delicately to reduce any physical damage. Microperfusion of squeezed or twisted slices was not performed.
- Microperfusion was performed with aCSF equilibrated with 5% CO₂ and 95% O₂ or with 95% N₂ and 5% CO₂. However, a measurement of the actual oxygen tension inside the microchamber was not possible. An attempt was made to use an oxygen sensor (Unisense, DK-8200 Aarhus N, Denmark) for this purpose, but without success, for the fine tip of the (expensive) sensor could not be placed in the microperfusion chamber without breaking.

- The weight (wet weight) of each brain slice (presented in table 3.3) was used to correct the final value of neurotransmitters levels in relation to slice size. The same flow rate (aCSF microperfusion flow) was used independently of the size of slices.

Slice vitality was confirmed by electrophysiological population recordings test (Appendix VII), meaning that all the potential sources of errors during each step, were minimized. No information is available to make an estimation of the number of alive cells present in the slice.

4.5 Release of neurotransmitters during hypoxia

The role of excitatory and inhibitory neurotransmitter in the brain in relation to anoxic/ischemic damage has been investigated already in some other different species (Nilsson, 1990, Nilsson et al., 1990, Lo et al., 1998, Lutz et al., 2003, Kirschner et al., 2009, Drew et al., 2013). Results from such studies are discussed below, under the relevant subheadings for the different compounds. But this is the first report of neurotransmitter efflux from the cerebellum in birds during modeled hypoxia. Temporal response during hypoxia exposure was investigated for the efflux of two neurotransmitters known to have an excitatory function in other species (Glu and Ser) (Lutz et al., 2003, Kirschner et al., 2009, Drew et al., 2013) and one with known primarily inhibitory function (Gly) (Foster and Kemp, 1989, Nilsson, 1990, Nilsson et al., 1990). Results for the Thr are also given even if the role of this amino acid related with hypoxia in the brain is still not well known. Kirschner et al. (2009) showed a significant increase of Thr levels in response to ischemia in Sprague Dawley rats (Kirschner et al., 2009).

Results for chicken show a generally significantly higher level of neurotransmitter efflux, both for control and treatment, compared with eider duck ($p < 0,05$) (tab. 3.4.5). As already previously explained, a measurement of the oxygen tension in the aCSF was not possible with the current instrumentation. One explanation could be given by the assumption of incomplete O₂ saturation in the normoxic aCSF, meaning that the normoxia status could already have been a weak hypoxia. Since the chicken brain tissue is not adapted to hypoxia in the same way as in the eider duck (Ludvigsen and Folkow, 2009), a possibly slightly hypoxic condition under “normoxic” perfusion could possibly be manifested with high levels of neurotransmitter, but as explained before, oxygen tension measurements that could support this hypothesis could not be made.

Besides, chicken slices were already proved by Ludvigsen & Folkow to be more hypoxia tolerant than e.g. rat slices (Ludvigsen and Folkow, 2009).

Temporal variation was not a significant factor, beside for the efflux of Glu in the eider ducks group, (F: 3,300_{5,41} p = 0,014) and for the efflux of Ser in the chickens group (F: 2,699_{5,41} p = 0,034), which showed a significant temporal fluctuation. However, only 6 pairwise comparisons between chicken and eider ducks have been considered, due to capacity limitations in the neurotransmitter analyses. More samples would need to be analysed to confirm/improve statistical analysis on potential temporal variations.

Neurotransmitter levels in some of the control groups decreased significantly over time compared with the treatment. This is probably a proof of tissue "ageing" inside the microperfusion chamber. A strong individual variation, given by large standard deviation (fig. 3.4.1 – 3.4.8), was present in both the species and for both treatment and control.

Glutamate

The level of Glu release from eider duck slices increased significantly in response to hypoxia, while during reoxygenation, Glu level fell below the baseline values (fig. 3.4.1 – 3.4.2). The latter may be a similar phenomenon as the observed slow decrease in Glu release from the control group over time (fig. 3.4.1 – 3.4.2), which could be related to the ageing of the preparation and drainage of the supply of Glu in the slice. Glu temporal efflux variation was significant only for the eider ducks (p = 0,014), with a significant increase of extracellular Glu level at minute 27, confirmed by pairwise comparison (tab. 3.4.2). Moreover, the chickens showed higher levels of Glu for both control and treatment when compared with the levels released from the eider ducks.

These results suggest that also in birds, enhanced Glu release under hypoxic conditions plays an important role and may contribute to excitotoxicity (Kirschner et al., 2009). Eider ducks, which are adapted to repeated shorts hypoxic conditions as a consequence of breath-hold diving, have probably delayed or attenuated release of this excitatory neurotransmitter, although this could not be conclusively demonstrated in the present study.

Knowledge on temporal response of Glu to model ischemia or hypoxia are available for small mammals (Lo et al., 1998, Kirschner et al., 2009, Drew et al., 2013), turtle (Nilsson et al., 1990) and fresh water fish (Nilsson, 1990).

In the fresh water turtle (*Pseudemys scripta elegans*), a species well-known to have a high tolerance to hypoxia (Lutz et al., 2003), Nilsson showed that the Glu level first increased, but then decreased by 24% after 4 hours and by 35% after 13 hours of anoxia (Nilsson et al., 1990). He

showed similar tendency in the crucian carp with a decrease by 49% after 18 days of hypoxia exposure (Nilsson, 1990).

Eider duck, in comparison, showed a slight increase in Glu release after the first minutes in hypoxia. These seabirds, even if very good divers (diving to less than 60 m) (Hawkins et al., 2000), are usually used to stay under the water for less than a minute (Guillemette et al., 2004). Therefore, they do not need to handle long-term hypoxic condition as crucian carps and fresh water turtles have to, but nevertheless may be challenged with hypoxemia on a regular basis, as shown for example for penguins (Ponganis et al 2007). Nevertheless, comparisons of temporal aspects of the Glu release between turtles/crucian carps and eider ducks are therefore difficult to make.

Comparison with small mammals that were subjected to acute hypoxia is probably more relevant. In Sprague Dawley rats (*Rattus norvegicus*) and the New Zealand white rabbit (*Oryctolagus cuniculus*), Kirschner and Lo showed that there was a large elevations in the Glu concentration during the first 10 minutes of ischemia (Lo et al., 1998, Kirschner et al., 2009). On the contrary, hippocampus slice of arctic ground squirrels showed delayed efflux of Glu during acute hypoxia (Drew et al., 2013). Such species was proved to be more resistant to hypoxia and ischemic-like condition, than hypoxia-vulnerable species such as rat.

Serine

Results for the eider ducks showed a significant increase in the level of Ser in response to hypoxia that persisted during the reoxygenation, as confirmed by pairwise statistical comparisons (fig. 3.4.5).

The chickens displayed higher levels of Ser for both control and treatment, compared to the eider ducks. Pairwise significant differences, between hypoxia and control, were only observed for chicken during reoxygenation, with a slight decrease in the Ser levels for the control group (fig. 3.4.6).

These results therefore suggest that also in birds Ser could act as a coagonist, at the "glycine site" (Shleper et al., 2005), available to activate the NMDA receptor and thereby contribute to excitotoxicity (Kirschner et al., 2009).

Ser efflux was found significantly elevated throughout the ischemic period in the rabbit cortex (Lo et al., 1998) and in rat hippocampus slices (Kirschner et al., 2009). In hypoxia tolerant species Ser release follows a different pattern from the Glu. Fresh water turtles showed an increasing in Ser

levels during hypoxia (Nilsson et al., 1990). In crucian carp, instead, Ser efflux remained unaltered after anoxia. Ser is the immediate precursor of Gly and it has some glycine-like actions (Nilsson, 1990).

Threonine

Thr increases in response to hypoxia in a manner similar to that of Ser in both eider duck and chicken (fig. 3.4.3 – 3.4.4). According to Kirschner et al. (2009), the chemical similarity between Thr and Ser suggests that these two amino acids use similar transport mechanisms. Kirschner et al. (2009) showed an increase of Thr levels in rat hippocampus slice during OD (Kirschner et al., 2009). The role of this compound as a neurotransmitter is still not clear. Further investigations are needed to clarify its role during OD.

Glycine

Gly increases significantly in response to OD in a manner similar to that of Ser and Thr in the eider ducks, confirmed also by pairwise comparison. In the chickens, Gly levels remained stable and decreased slightly under control conditions (fig. 3.4.7 – 3.4.8).

In mammals the role of this neurotransmitter during anoxia is ambiguous, having both inhibitory and excitatory properties (Foster and Kemp, 1989). Even though in the mammalian neocortex Gly is considered an allosteric activator of the glutamate receptors (NMDA), it is well established as an inhibitory neurotransmitter in lower brain areas (Lutz et al., 2003). Gly is an antagonist of the D-ser (“Gly”) binding site and is neuroprotective in animal models of stroke (Wasterlain et al., 1996, Ohtani et al., 2000, Ohtani et al., 2003). In hypoxia tolerant species, as turtles and crucian carps, inhibitory neurotransmitters are released in large amounts during hypoxia exposure. They act by depressing neural activity and hence ATP use and thereby protect (Nilsson, 1990, Nilsson et al., 1990). Nilsson showed that anoxia produces increased intracellular Gly concentrations in both the fresh water turtle (Nilsson et al., 1990) and the crucian carp (Nilsson, 1990) brain. In the rat, by contrast, no increase of Gly was seen during ischemia (Erecinska et al., 1984) which perhaps indicates an important difference in the inhibitory role of Gly between the anoxia-tolerant brain and the mammalian brain. The fact that the release of Gly levels increased significantly in eider duck slices but not in chicken slices may indicate that Gly release is enhanced in the divers, possibly reflecting a neuroprotective role.

GABA is also a well-established inhibitory neurotransmitter, probably involved in the depression of the brain metabolism (Lutz et al., 2003). Surprisingly, it cannot be seen in any of the samples analysed by UPLC, probably because of its too low concentration.

4.6 Ethics

Eider ducks were kept in the research animal facilities at the ABB of UiT The Arctic University of Norway. A suitable in and outdoor facility with a small pool, during the summer and the winter season, ensured the well-being of these animals. Chickens were hatched and reared in similar conditions by a private owner, from whom they were purchased. Altogether 10 birds (6 eider ducks and 4 chickens) were euthanized to investigate the role of neurotransmitter efflux from cerebellum slice during hypoxia. This is a minimum number to obtain scientifically and statistically valid information and if available, it would have been desirable to increase the number of chickens. An appropriate anesthetic (intramuscular injection of Ketalar (ketamine hydrochloride 50 mg/ml, Pfizer Inc., New York, USA) and Xylazin (Rompun vet. xylazin chloride 20 mg/ml, Bayer AG, Leverkusen, Germany) (Green et al., 1981)) was used to avoid potential distress and pain during the handling procedures and before euthanasia.

Live brain tissue was required to measure the release of neurotransmitters during simulated hypoxia.

Nowadays, there are a number of alternative methods that can be used to replace the use of living animals. Recently, studies of the brain have improved enormously through the use of in vivo and in vitro models. Some future prospects could possibly be addressed in brain cell cultures, or even dissociated cultures of microglia, astrocytes, oligodendrocytes and neurons (Gibbons and Dragunow, 2010), while others require intact tissue in order to allow the interplay between different cells to be studied.

4.7 Conclusion

The developed method was validated with respect to specificity, linearity, quantification and precision. The Waters MassTrak™AAA kit proved to be a robust, reliable, and reproducible method. Results demonstrate that the modifications of the method (e.g. run time) made it more effective in reducing the costs and the time of analysis.

Ultra-Performance Liquid Chromatographic (UPLC) analysis of amino acids in aCSF from cerebellum slices of eider ducks and chickens showed results that can be compared with similar studies done on other species (Nilsson, 1990, Nilsson et al., 1990, Lo et al., 1998, Lutz et al., 2003, Kirschner et al., 2009, Drew et al., 2013).

In agreement with previous studies (Ludvigsen and Folkow, 2009), results for eider duck and chicken implied a higher hypoxia tolerance since it was observed a significantly blunted release of excitatory transmitters (Glu and Ser). However, is not confirmed that the brain neurons of the eider duck display a higher hypoxia tolerance than those of chicken, since no visible significantly blunted release of excitatory transmitters (Glu and Ser), and/or a significantly enhanced release of Gly in ducks compared to chicken could be conclusively confirmed. Besides, a generally higher level of neurotransmitter efflux, both for control and treatment, was observed in chicken compared with the eider duck.

There was a high variation between individuals of the same species and this is possibly due to differences in the vitality and anatomy of the single brain slices.

Future prospective

Stroke is one of the most leading causes of death in humans. The resulting brain damage by stroke is a consequence of an insufficient blood supply to the brain, followed by a cascade of excitotoxic events as a result of insufficient oxygen supply (Ramirez et al., 2007). The hippocampus is considered as highly susceptible to global cerebral ischemia experienced during cardiac arrest (Kirino, 2000).

Recently, brain neurons of the hooded seal (*Cystophora cristata*) have been shown to be more hypoxia tolerant than those of small mammals as the mice (Folkow et al., 2008). Microperfusion tests on visual cortex and hippocampus slice of hooded seals have also been performed in parallel with the presently described study in eider ducks and chickens. However, time and costs have delayed the analysis with the Ultra-Performance Liquid Chromatography and suitable control experiments with representative non-diving mammalian species have not yet been conducted. Efflux of neurotransmitter patterns, under hypoxia conditions, deserve to be investigated especially in a large diving mammal as the hooded seal, already tested for its extraordinary ability to tolerate hypoxia (Czech-Damal et al., 2014).

In addition, more investigations should be conducted on others neurotransmitter that have a proved important role in the ischemic brain (e.g. GABA) (Richter et al., 1999, Lutz et al., 2003).

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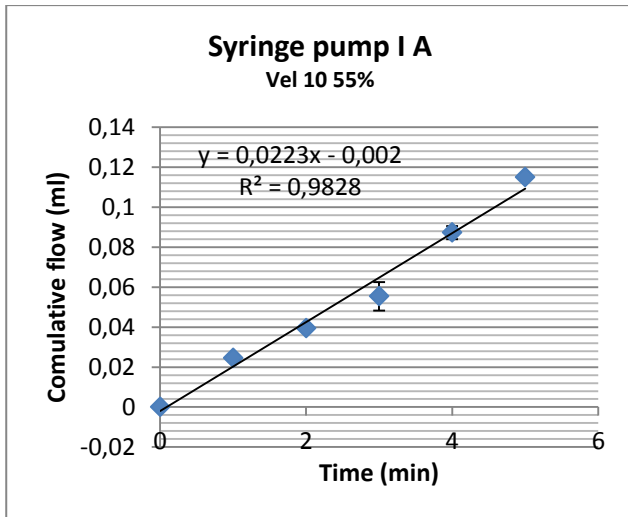
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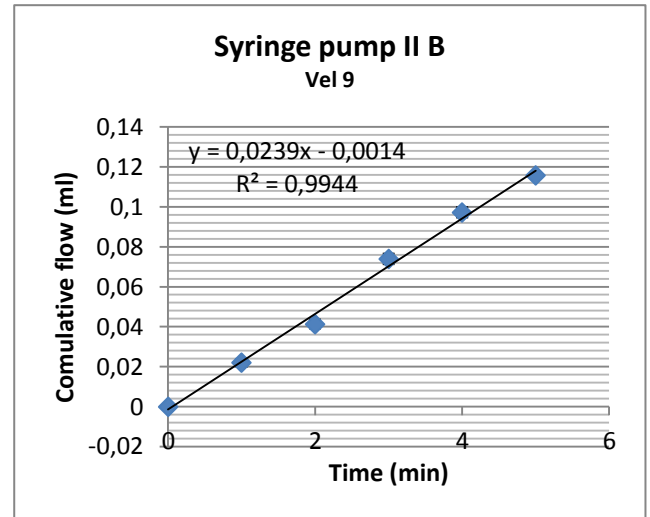
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Appendix

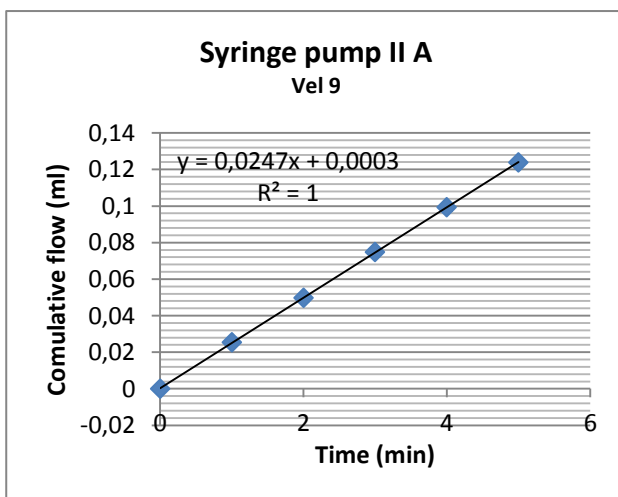
Appendix I :



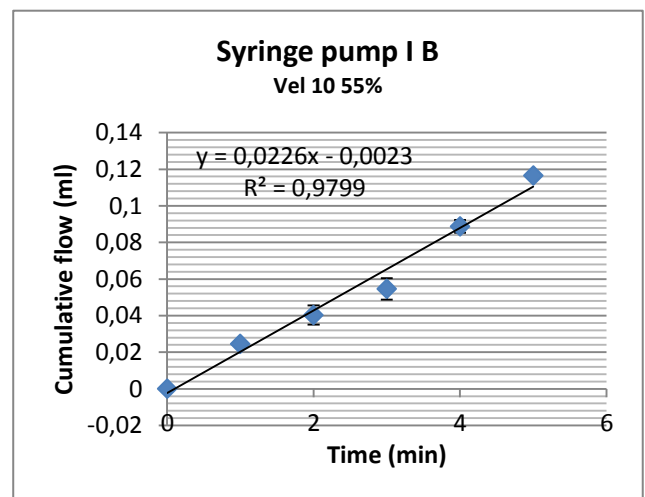
Flow rate syringe pump I A = 0,024 ml/min



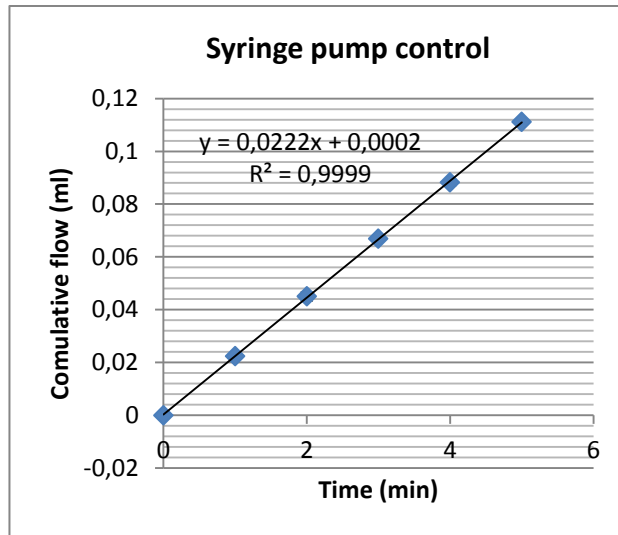
Flow rate syringe pump I B = 0,024 ml/min



Flow rate syringe pump II A = 0,025 ml/min



Flow rate syringe pump II B = 0,022 ml/min



Flow rate syringe pump control = 0,022 ml/min

Appendix II :

Waters Acquity SDS Method

Solvent C Name: Water
 Solvent D Name: Acetonitrile
 Low Pressure Limit: 0 psi
 High Pressure Limit: 15000 psi
 Seal Wash Period: 5.00 min
 [Gradient Table]

Time(min)	Flow Rate(mL/min)	%A	%B	%C	%D	Curve
1. Initial	0.400	0.0	0.0	99.9	0.1	Initial
2. 1.14	0.400	0.0	0.0	99.9	0.1	6
3. 5.00	0.400	0.0	0.0	98.5	1.5	6
4. 10.00	0.400	0.0	0.0	95.0	5.0	6
5. 20.00	0.400	0.0	0.0	80.0	20.0	6
6. 25.00	0.400	0.0	0.0	2.0	98.0	6
7. 28.00	0.400	0.0	0.0	2.0	98.0	6
8. 28.10	0.400	0.0	0.0	99.9	0.1	6
9. 32.00	0.400	0.0	0.0	99.9	0.1	6

Flow Ramp Rate: 0.45 min

Run Time: 32.00 min
 Load Ahead: Disabled
 Loop Offline: Automatic min
 Wash Solvent Name: Water
 Pre-Inject Wash Time: 6.0 sec
 Post-Inject Wash Time: 6.0 sec
 Purge Solvent Name: Water
 Dilution: Disabled
 Dilution Volume: 0 uL
 Delay Time: 0 min
 Dilution Needle Placement: 4 mm
 Target Column Temperature: Off C
 Target Sample Temperature: Off C
 Sample Temperature Alarm Band: 25.0 C
 Syringe Draw Rate: 30 uL/min
 Needle Placement: Automatic

Pre-Aspirate Air Gap: Automatic
Post-Aspirate Air Gap: Automatic

Waters Acquity PDA Method

Run Time: 32.00 min
PDA Detector Type: UPLC LG 500 nm
Lamp: On
Sampling Rate: 20 points/sec
Filter Time Constant: 0.1000 sec
Exposure Time: Auto msec
Negative Absorbance Margin: -0.07 AU
Interpolate 2nd order filter (340nm) Region: No
Use UV Blocking Filter: No
Range: 210 - 500
Resolution: 1.2 nm
Initial Switch 1: No Change
Pulse Width: 1.0 sec
Rect Wave Period: 0.2 sec

Waters Acquity CM Method

Target Column Temperature: 43.0 C
Column Temperature Alarm Band: 2.0 C
Column Valve Position: Column 1

Appendix III:

List of Chemicals

D-Norvaline, Synonym: (R)-(-)-2-Aminopentanoic acid, 99% code 851620, Sigma-Aldrich Co. LLC
St. Louis, Missouri

Ketalar, ketamine hydrochloride 50 mg/ml, Pfizer Inc., New York, USA

Xylazin, Rompun vet. xylazin chloride 20 mg/ml, Bayer AG, Leverkusen, Germany

AccQ•Tag Ultra Derivatization Kit, 250 analyses [186003836] Instruction manual:

<http://www.waters.com/webassets/cms/support/docs/715001331.pdf>

AccQ-Tag Eluent A [WAT052890]

AccQ-Tag Ultra Eluent B [186003839]

Amino Acid Hydrolysate Standard, 10 x 1 mL [WAT088122]

Appendix IV:

Cleaning protocol: One-time cleaning (enough to be performed only once)

Cleaning solvents:

- A) 50:50 (v/v) methanol : water
- B) 30:70 (v/v) phosphoric acid : water
- C) 100% water
- D) 100% isopropanol

To clean the system:

- 1) Place all lines A1, A2, B1, B2 seal wash, and strong needle wash into A) 50:50 methanol : water.
- 2) Prime the solvent lines for 5 minutes each.
- 3) Prime the seal wash.
- 4) Prime the wash syringes and sample syringe for 4 cycles.
- 5) Connect a pressure restrictor in the fluid path after the injector to create 2000 psi backpressure in the system.
- 6) Transfer 1 mL of mobile phase to an auto sampler vial and place it in position 1:A, 1.
- 7) Create an instrument method with the following parameters:
 - Flow rate = 0.5 mL/min
 - Gradient composition 50% A1:50% B1
 - Full loop injection (2X overfill)
- 8) Make 30 full loop injections from the vial containing the mobile phase. Set the run time to 0.5 minutes.
- 9) Repeat steps 1 through 8 using 100% isopropanol as the solvent.

Restriction: Do not pass effluent through the optical detector for this wash step. Route the restrictor to waste
- 10) Repeat steps 1 through 8 using 100% water as the solvent.
- 11) Repeat steps 1 through 8 using 30:70 (v/v) phosphoric acid : water as the solvent.
- 12) Repeat steps 1 through 8 using 100% water as the solvent.
- 13) Repeat steps 1 through 8 using 50:50 (v/v) methanol : water as the solvent.

Tip: This test takes approximately 6 hour.

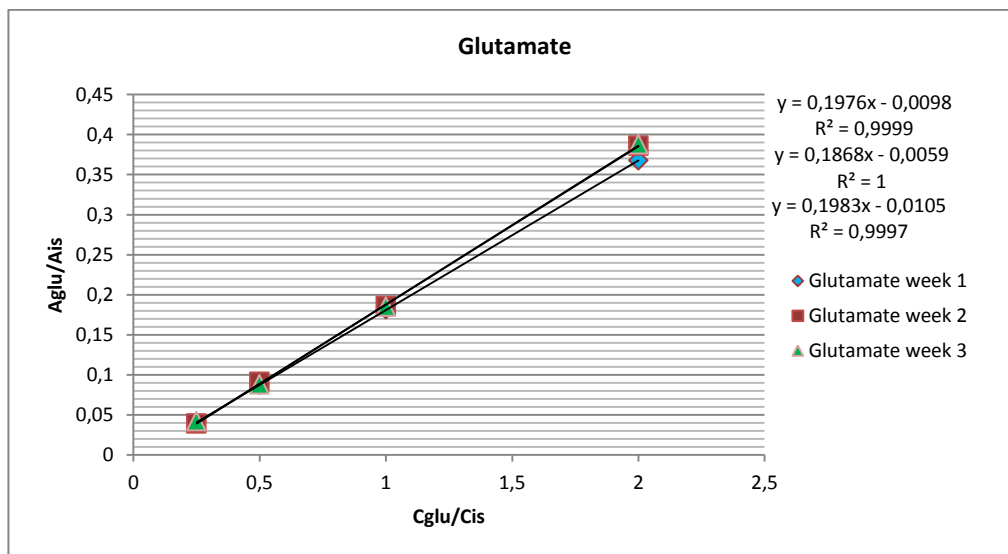
Cleaning protocol: Each time cleaning (to be performed before start to run a new set of samples)

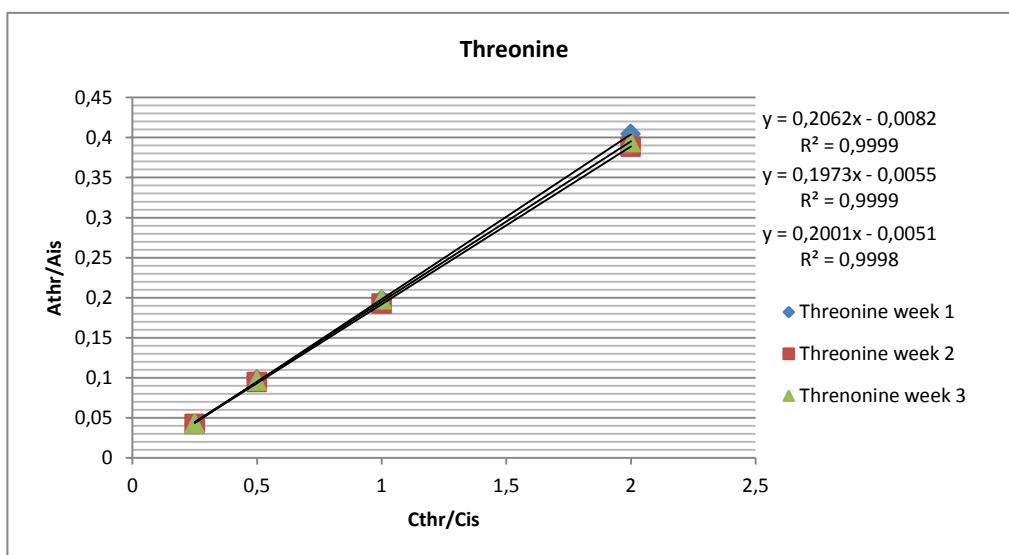
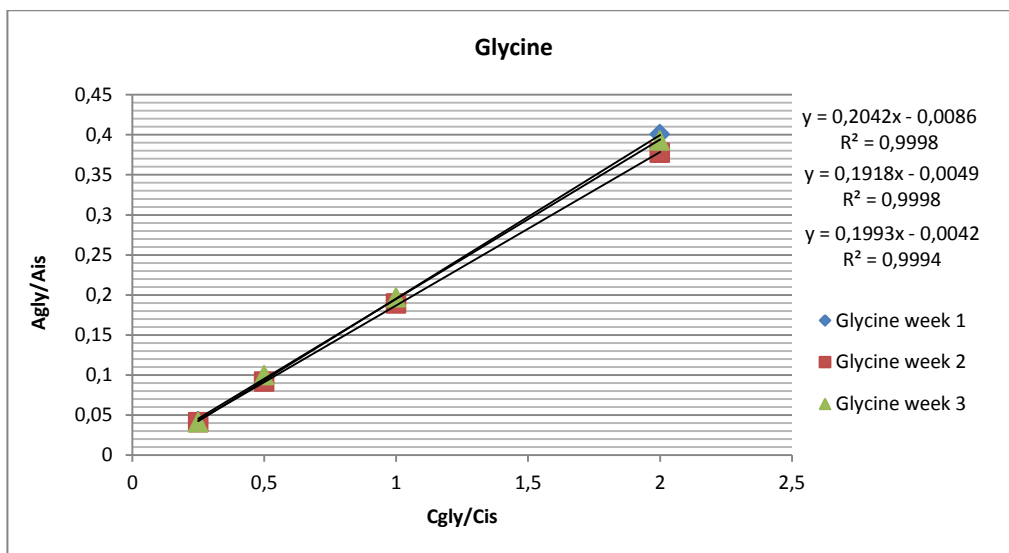
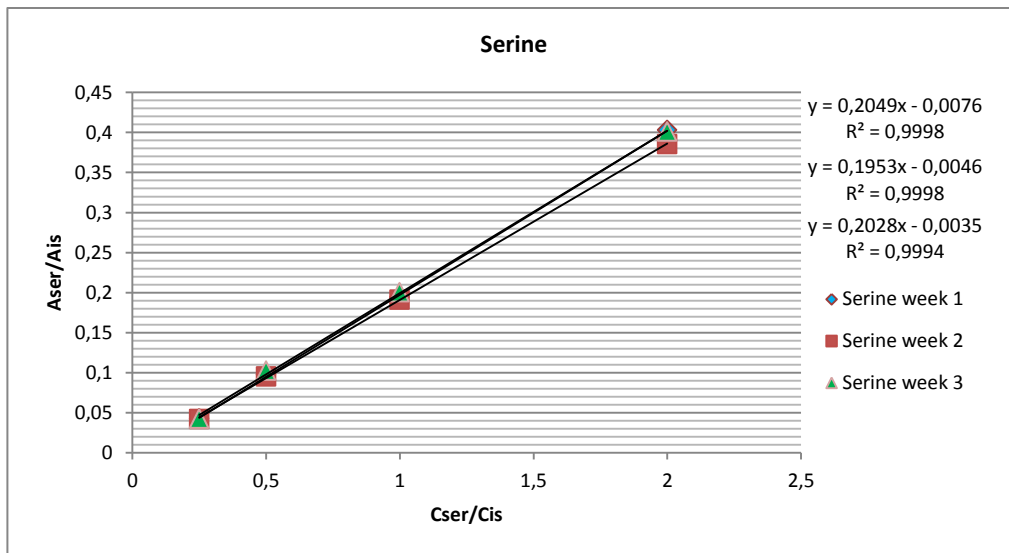
- 1) Install Eluent A on line A1 and Eluent B on lines B1 and B2.
- 2) Install the Seal Wash line into a 80:20 (v/v) water/acetonitrile solution.
- 3) Install the Strong Needle Wash line into a 95:5 (v/v) acetonitrile/water solution.
- 4) Prime all solvent lines for 5 minutes.
- 5) Prime wash/sample syringes for 4 cycles.
- 6) Run the method for at least 30 minutes.

Tip: This test takes approximately one hours.

Appendix V:

Calibration curve





Appendix VI :

Statistical analysis

Linear Mixed Model based on the Bonferroni correction used to determine significant differences between treatment and control (Exp).

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	41	384.648	.000
Exp	1	41	25.735	.000
Time	5	41	3.300	.014

a. Chemical = glutamate, Species = Eider

The F tests the effect of Exp. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	46	555.228	.000
Exp	1	46	4.862	.032

a. Chemical = glutamate, Species = Chicken

The F tests the effect of Exp. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	46	2566.966	.000
Exp	1	46	55.678	.000

a. Chemical = threonine, Species = Eider

The F tests the effect of Exp. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	46	1427.406	.000
Exp	1	46	25.480	.000

a. Chemical = threonine, Species = Chicken

The F tests the effect of Exp. This test is based on the linearly independent pairwise comparisons among the estimated marginal means

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	46	1915.563	.000
Exp	1	46	66.995	.000

a. Chemical = serine, Species = Eider

The F tests the effect of Exp. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	41	849.116	.000
Exp	1	41	40.898	.000
Time	5	41	2.699	.034

a. Chemical = serine, Species = Chicken

The F tests the effect of Exp. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	46	2140.438	.000
Exp	1	46	109.735	.000

a. Chemical = glycine, Species = Eider

The F tests the effect of Exp. This test is based on the linearly independent pairwise comparisons among the estimated marginal means

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	46	1415.531	.000
Exp	1	46	24.039	.000

a. Chemical = glycine, Species = Chicken

The F tests the effect of Exp. This test is based on the linearly independent pairwise comparisons among the estimated marginal means

Linear Mixed Model based on the Bonferroni correction used to determine significant differences in the effect of treatment, between the eider duck and chicken group.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	86	540.747	.000
Species	1	86	18.966	.000

a. Chemical = glutamate, Exp = control

The F tests the effect of Species. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	41	559.197	.000
Time	5	41	3.230	.015
Species	1	41	5.439	.025

a. Chemical = glutamate, Exp = hypoxia

The F tests the effect of Species. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	86	4065.045	.000
Species	1	86	39.572	.000

a. Chemical = threonine, Exp = control

The F tests the effect of Species. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	86	2894.236	.000
Species	1	86	24.107	.000

a. Chemical = threonine, Exp = hypoxia

The F tests the effect of Species. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	86	1697.683	.000
Species	1	86	5.513	.021

a. Chemical = serine, Exp = control

The F tests the effect of Species. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	86	2115.913	.000
Species	1	86	14.246	.000

a. Chemical = serine, Exp = hypoxia

The F tests the effect of Species. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	86	3091.495	.000
Species	1	86	59.115	.000

a. Chemical = glycine, Exp = control

The F tests the effect of Species. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	85	2277.422	.000
Species	1	85	4.770	.032

a. Chemical = glycine, Exp = hypoxia The F tests the effect of Species.

This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Statistics for pairwise comparison

Only one example is here attached: Chemical = glutamate, minute = 27, Species = Eider

Descriptive Statistics

Exp	Count	Mean	Standard Deviation	Coefficient of Variation
control	4	353.724620407 111600	151.626982843 773500	42.9%
hypoxia	4	679.865708118 335600	213.288262984 366900	31.4%
Total	8	516.795164262 723600	244.418813090 068280	47.3%

Model Dimension^a

		Number of Levels	Number of Parameters
Fixed Effects	Intercept	1	1
	Exp	2	1
Residual			1
Total		3	3

a. Chemical = glutamate, Time = 27:00, Species = Eider

Tests of Fixed Effects^a

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	6	62.399	.000
Exp	1	6	6.213	.047

a. Chemical = glutamate, Time = 27:00, Species = Eider

Pairwise Comparisons^a

(I) Exp	(J) Exp	Mean Difference (I-J)	Std. Error	df	Sig. ^d	95% Confidence Interval for Difference ^d	
						Lower Bound	Upper Bound
control	hypoxia	-326.141 [*]	130.846	6	.047	-646.310	-5.973
hypoxia	control	326.141 [*]	130.846	6	.047	5.973	646.310

Based on estimated marginal means

*. The mean difference is significant at the ,05 level.

a. Chemical = glutamate, Time = 27:00, Species = Eider

d. Adjustment for multiple comparisons: Bonferroni.

Appendix VII : Typical population recordings cerebellum from brain slices of eider ducks and chickens.

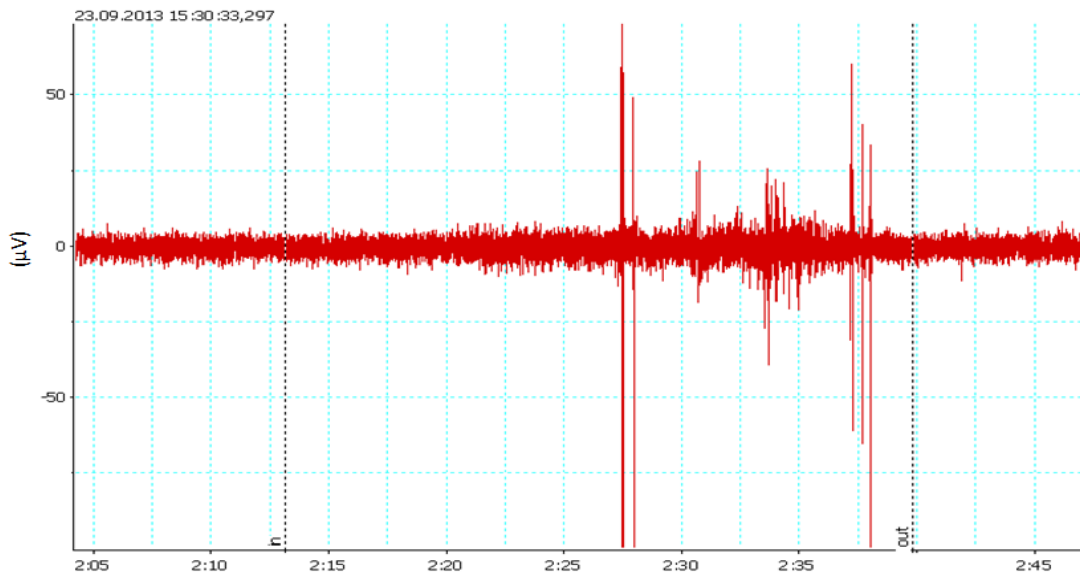


Figure A: A typical recording cerebellum slice of a chicken, after 20 minutes hypoxia treatment. In: Stimulator in contact with the cerebellum slice. Out: The stimulator is not touching the cerebellum slice. X-axis: Time recording. Y-axis: Membrane potential (μV).

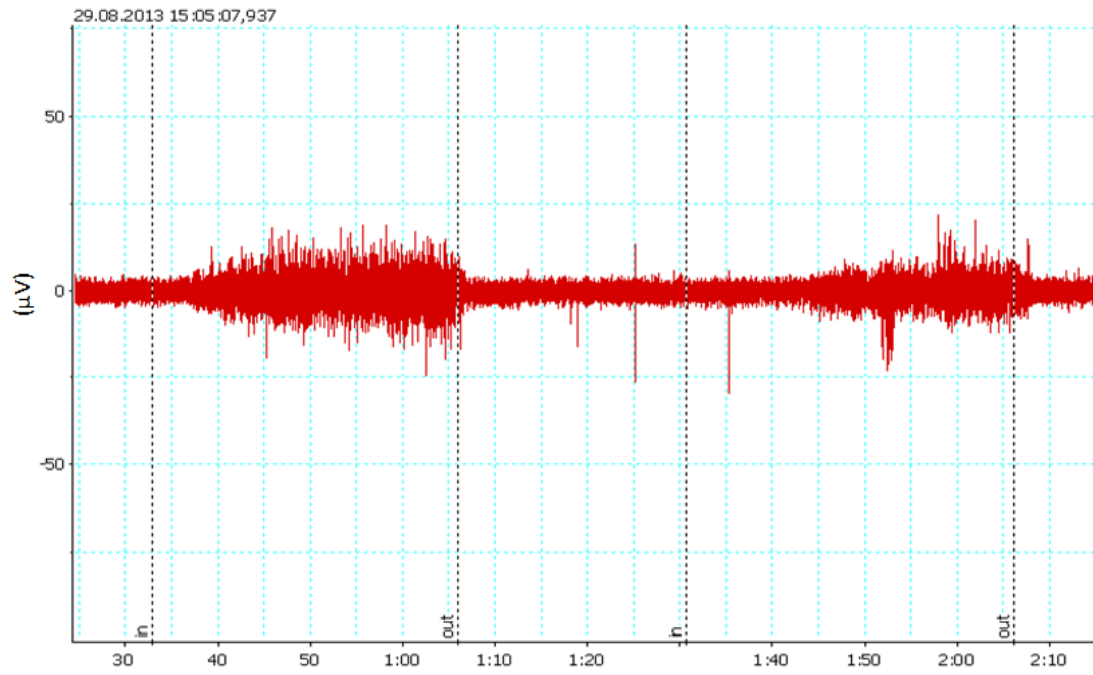


Figure B: A typical recording cerebellum slice of an eider duck, after 20 minutes hypoxia treatment. In: Stimulator in contact with the cerebellum slice. Out: The stimulator is not touching the cerebellum slice. X-axis: Time recording. Y-axis: Membrane potential (μV).