Glutamate Turnover and Energy Metabolism in Brain Injury

Clinical and Experimental Studies

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Abstract

During brain activity neurons release the major excitatory transmitter glutamate, which is taken up by astrocytes and converted to glutamine. Glutamine returns to neurons for re-conversion to glutamate. This glutamate-glutamine cycle is energy demanding. Glutamate turnover in injured brain was studied using an animal iron-induced posttraumatic epilepsy model and using neurointensive care data from 33 patients with spontaneous subarachnoid hemorrhage (SAH). Immunoblotting revealed that the functional form of the major astrocytic glutamate uptake protein GLT-1 was decreased 1-5 days following a cortical epileptogenic iron-injection, presumably due to oxidation-induced aggregation. Using microdialysis it was shown that the GLT-1 decrease was associated with increased interstitial glutamate levels and decreased interstitial glutamine levels. The results indicate a possible posttraumatic and post-stroke epileptogenic mechanism. Analysing 3600 microdialysis hours from patients it was found that the interstitial lactate/pyruvate (L/P) ratio correlate with the glutamine/glutamate ratio (r = -0.66). This correlation was as strong as the correlation between L/P and glutamate (r=0.68) and between lactate and glutamate (r=0.65). Pyruvate and glutamine correlated linearly (r=0.52). Energy failure periods, defined as L/P>40, were associated with high interstitial glutamate levels. Glutamine increased or decreased during energy failure periods depending on pyruvate. Energy failure periods were clinically associated with delayed ischemic neurological deficits (DIND) or development of radiologically verified infarcts, confirming that L/P>40 is a pathological microdialysis pattern that can predict ischemic deterioration after SAH. DIND-associated microdialysis patterns were L/P elevations and surges in interstitial glutamine. Glutamine and pyruvate correlated with the cerebral perfusion pressure (r=0.25, r=0.24). Glutamine and the glutamine/glutamate ratio correlated with the intracranial pressure (r=0.29, r=0.40). Glutamine surges appeared upon substantial lowering of the intracranial pressure by increased cerebrospinal fluid drainage. Increased interstitial glutamine and pyruvate levels may reflect augmented astrocytic glycolysis in recovering brain tissue with increased energy demand due to a high glutamate-glutamine turnover.

Keywords: glutamate, glutamine, lactate, pyruvate, microdialysis, GLT-1, energy metabolism, intracranial pressure, cerebral perfusion pressure, subarachnoid hemorrhage, ischemia, epilepsy

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List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


IV Samuelsson C, Hillered L, Kumlien E, Enblad P, Ronne-Engström E. Microdialysis patterns in subarachnoid hemorrhage patients with focus on ischemic events and brain interstitial glutamine levels. Manuscript


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Abbreviations

AMPA: $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ATP: adenosine triphosphate
BBB: blood-brain barrier
BP: blood pressure
CBF: cerebral blood flow
CPP: cerebral perfusion pressure
CSF: cerebrospinal fluid
CT: computed tomography
EAA: excitatory amino acids
EAAT: excitatory amino acid transporter
EEG: electroencephalogram
GFAP: glial fibrillary acidic protein
GLAST: glutamate and aspartate transport protein
Gln: glutamine
Glt: glutamate
GLT-1: glutamate transporter-1
GS: glutamine synthetase
HPLC: high performance liquid chromatography
ICH: intracerebral hemorrhage
ICP: intracranial pressure
i.p.: intraperitoneally
L/P: lactate/pyruvate
mBP: mean blood pressure
MD: microdialysis
NICU: neurointensive care unit
NMDA: N-methyl-D-aspartate
PAG: phosphate activated glutaminase
PBN: $\alpha$-phenyl-tert-N-butyl nitrone
PVDF: polyvinylidene difluoride
RLS 85: Reaction Level Scale 85
SAH: subarachnoid hemorrhage
SC: subcutaneous
SD: standard deviation
SE: standard error of the mean
SNAT 1-2: system A amino acid transporter
SNAT 3: system N amino acid transporter
TBI: traumatic brain injury
TCA: tricarboxylic acid
VSP: vasospasm
WFNS: World Federation of Neurosurgical Surgeons
Introduction

Stroke and traumatic brain injury (TBI) are severe acute brain insults where secondary pathophysiological processes such as focal or global ischemia, altered energy metabolism, increased intracranial pressure (ICP) and excitotoxicity are commonly initiated. Neurointensive care aims at preventing loss of viable brain tissue due to these secondary processes which are believed to contribute significantly to morbidity and disability. Since TBI and stroke have high mortality rates, often cause long-lasting functional impairment in survivors and have great social and economic impact much can be gained if the brain damage progression can be hampered. To achieve this, profound knowledge about what goes on in the injured brain is required. This thesis is an attempt to improve the understanding of some aspects of the cerebral glutamate turnover, which appears to play a role in the pathophysiological processes following acute brain injury.

Acute brain injuries

Epidemiology

Stroke, which includes ischemic cerebral infarct (85%), intracerebral hemorrhage (ICH) (10%) and subarachnoid hemorrhage (SAH) (5%) annually strike approximately 20,000 persons in Sweden. National and international data indicate that around 250/100 000 are annually hospitalized due to TBI. Severe TBI, which often requires surgical intervention, accounts for 4-10% of all TBI cases.

Neurointensive monitoring and care

Patients with acute brain injuries, typically TBI, SAH or ICH, are often admitted to the neurointensive care unit (NICU) where aggressive monitoring and optimization of physiological variables aim at promoting recovery and improve outcome.

Since NICU patients often are sedated and/or have decreased consciousness they commonly require ventilatory support to assure adequate oxygenation. A major focus is placed on meticulous observation of the patients’ neu-
logical state and physiological parameters, including blood pressure (BP), ICP, cerebral perfusion pressure (CPP), SpO₂, arterial gases, electrolytes and temperature, in order to detect preventable deteriorations and initiate diagnostic procedures and treatment.

Brain blood flow and energy metabolism

The brain has high energy demands: it represents 2% by weight, uses 20% of the oxygen and glucose supply, and receives 15% of the cardiac output in the resting adult body. Cerebral blood flow (CBF) normally remains relatively unaltered in the mean arterial blood pressure (mBP) range 60-150 mmHg, owing to an ability of the cerebral arterioles and precapillary sphincters to vary their resistance. This mechanism is referred to as “cerebral autoregulation” and ensures a sufficient supply of oxygen and nutrients to the brain without exposing it to unnecessary large blood volumes. However, in traumatized or ischemic brain, the autoregulatory mechanism can be malfunctioning. As a consequence, CBF becomes increasingly blood pressure dependent, why hypovolemia and/or hypotension can have deleterious effects. The CBF can also be reduced as a consequence of intracranial expansive processes (e.g. hematoma, edema or hydrocephalus) which elevate the ICP.

Glucose is the brain’s main energy substrate. Adenosine triphosphate (ATP) is produced almost exclusively by oxidative glucose metabolism, which is tightly linked to brain activity. In the resting state both astrocytes and neurons can take up glucose and completely oxidize it to CO₂ and H₂O, which yields an oxygen/glucose ratio that approaches six. During brain activity, astrocytes are however capable of a several-fold increase in glycolysis as a response to an increased energy demand. If there is a high glycolytic rate pyruvate and reduced nicotinamide adenine dinucleotides (NADH) will accumulate. To regenerate NAD⁺ (oxidized form of NADH) and restore the intracellular re-dox potential, pyruvate will be reduced to lactate by lactate dehydrogensase (LDH). Monocarboxylates (lactate and pyruvate) can be shuttled from astrocytes to neurons via the interstitial space and neurons are capable of switching between glucose and monocarboxylates as oxidation substrates, depending on the relative availability in the interstitial space. This compartmentalization of anaerobic and aerobic glucose metabolism between astrocytes and neurons in situations of high cerebral metabolic demands has found increasing support during the last decade.
Ischemia

Ischemia is a relative shortage of the blood supply, which results in inadequate oxygenation and glucose delivery to the tissue. Ischemia can consequently occur as a result of decreased delivery of oxygenated blood or increased demand of oxygenated blood. During ischemia there is progressive ATP production failure in the cells with associated breakdown of mechanisms necessary for cellular function (functional ischemia) and survival (structural ischemia/infarct). Lack of oxygen at the mitochondrial level as well as mitochondrial inability to utilize oxygen, will lead to NADH accumulation, reduced cellular redox-state, and increased lactate production from pyruvate.

Stroke and TBI patients are at high risk of brain energy failure and ischemia. Common systemic complications, such as hypoxia, hypotension, epileptic seizures and fever, can result in a mismatch between brain oxygen supply and demand. In addition, there may be spontaneous, post-operative or traumatic lesions such as hematoma, contusion or edema, which further compromise the blood delivery to adjacent regions.

Ischemia in SAH

Following a SAH there are a number of specific, primary and secondary, causes of ischemic brain damage. When a cerebral artery ruptures the ICP can approach and exceed mBP levels, resulting in cerebral circulatory arrest and global brain ischemia with unconsciousness at the onset of the bleed. In the sub-acute phase following SAH there is a high risk of ischemia due to vasospasm (VSP), which is a seemingly non-purposeful constriction of the basal cerebral arteries. Using angiography, VSP is diagnosed in 60-70% of all SAH patients where narrowed segments of large caliber cerebral arteries can be visualized. However, only 20-40% of all SAH patients present with clinical symptoms of VSP, which include various degrees of neurological deterioration attributed to ischemia. The incidence of VSP is at highest 4-10 days post SAH and risk factors include large amount of blood and bilirubin oxidation products in the subarachnoid space. Calcium antagonists, such as nimodipine which is believed to mediate its effects through neuroprotection and cerebral vasorelaxation, is routinely used as VSP prophylaxis in SAH patients. Delayed ischemic neurological deficits (DIND) following SAH are often attributed to VSP but may also be a consequence of interacting cerebral and/or systemic secondary complications. In case of DIND due to VSP, in the absence of structural (radiologically verified) ischemia, hypertensive-, hypervolemic-, hemodilution-therapy is normally initiated to improve cerebral blood flow and perfusion.
Seizures

A seizure is the clinical manifestation of abnormal synchronized neuronal discharge. Epilepsy is diagnosed when seizures appear repeatedly and/or unprovoked. In partial epilepsy, where seizure activity originates from a definite region in the brain, there is frequently an identifiable cause in the past, e.g. TBI, status epilepticus, stroke, brain tumor or an infection. It appears as such insults can trigger cascades of neurochemical events, including generation of free radicals, leading to spontaneous seizures, referred to as remote symptomatic seizures, after a latency period. This latency period, corresponding to the epileptogenesis, normally ranges from months to years.

Seizures are common within the first week after TBI and stroke, and are feared complications as they exert increased metabolic demands on an already vulnerable brain. Occurrence of acute seizures are prognostic of increased risk for developing chronic epilepsy. Prophylactic administration of anti-epileptic drugs reduces the incidence of early seizures, but has neither lead to increased survival nor to reduced incidence of remote symptomatic seizures. This indicates that early seizures are a manifestation of ongoing epileptogenic processes in the injured brain but that an early seizure activity per se does not contribute to epileptogenesis.

The relative risk of developing epileptic seizures after stroke or TBI, compared to a non-stroke/TBI population, is between 1–50 % depending on the severity and type of insult. Epilepsy is often a major component of disability and contributes to pre-mature death in patients who have survived TBI and stroke. Presence of early seizures, focal cortical damage, skull fractures, loss of consciousness, age, computed tomography (CT) - verified infarcts, cortical depositions of hemosiderin and blood in the subdural/subarachnoid space are risk factors for developing epilepsy following stroke and TBI.

Glutamatergic neurotransmission

The main excitatory transmitter glutamate binds to postsynaptic ligand-gated ion channels (NMDA-, AMPA- and kainate receptors), which leads to neuronal excitation through depolarization. The receptor activation is normally transient but if it becomes excessive the target neuron will die. Such glutamate-induced cell death is called excitotoxicity and involves prolonged depolarization, ionic imbalance, ATP depletion and elevations of the intracellular free calcium concentration.

The average level of glutamate in the brain is ca $\pm 10$ mmol/kg but the transmitter is normally heavily compartmentalized in order to achieve a high
signal to noise ratio in the synaptic cleft. With microdialysis (MD) the basal interstitial glutamate concentration in human brain has been reported to be \( \sim 15 \, \mu M \) \( ^{145,165} \) and most animal studies report concentrations in the low micromolar range \( ^{5,103,119} \). There is however evidence that the NMDA receptor is activated by glutamate levels below 5 \( \mu M \) range \( ^{128} \), which, together with experimental evidence \( ^{75} \), implies that the resting synaptic glutamate concentration in normal brain is below this level.

**Glutamate uptake**

High affinity, cell membrane bound, excitatory amino acid transporters (EAATs) are responsible for the removal of glutamate from the interstitial space and thereby aid in terminating synaptic transmission and prevent synaptic glutamate concentrations from rising to neurotoxic levels. Five different EAATs have been identified \(^4\). The most abundant and functionally most important was in mouse originally named glial glutamate transporter 1, GLT-1 (human homologue is called EAAT-2) \(^{148,154}\). It is localized predominately on astroglial processes \(^{42}\). GLT-1 accounts for some 90% of the glutamate uptake in the forebrain \(^{71}\). The transport of glutamate into astrocytes requires energy and is driven by the co-transport of \( Na^+ \) and counter-transport of \( K^+ \) down their respective concentration gradients (Figure 1).

![Figure 1](image.png)

*Figure 1.* The astrocytic uptake of glutamate through GLT-1 is driven by flow of \( Na^+ \) and \( K^+ \) down their respective concentration gradients. The membrane potential is restored thanks to the action of \( Na^+/K^+ \) ATPase. Glt: glutamate, Gln: glutamine, GLT-1: glutamate transporter 1.

Upon dissipation of the electrochemical gradient across the cell membrane, e.g. during ischemia and energy failure, the GLT-1 mediated glutamate transport is impaired or even reversed with release of glutamate instead of uptake \(^{182}\).
The glutamate-glutamine cycle

After astrocytic uptake, glutamate is converted to glutamine by the ATP-dependent glia-specific enzyme glutamine synthetase (GS) which adds an amine group from ammonia to the glutamate carbon skeleton. The ammonia derives from the blood or from the brain’s metabolism. Astrocytes release glutamine back to the interstitial space via SNAT 3, a system N amino acid transporter\(^\text{18,21}\). Neuronal uptake of glutamine occurs predominantly via system A amino acid transporters SNAT 1 and SNAT 2\(^\text{21,46,87,173}\), but other transporters may also be involved in neuronal glutamine uptake\(^\text{38}\). SNAT 3 can carry glutamine bi-directionally across the astrocytic cell membrane and glutamine efflux is favored by low extracellular pH\(^\text{20}\). The intracellular glutamine concentration in both astrocytes and neurons normally exceeds the interstitial concentration\(^\text{22,127}\), i.e. glutamine exits astrocytes down a concentration gradient and enters neurons against a concentration gradient. Inside the neuron glutamine is re-converted into glutamate by mitochondrial phosphate activated glutaminase (PAG) in order to replenish the pre-synaptic glutamate transmitter pool\(^\text{40}\). This efficient uptake and regeneration of glutamate and glutamine between neurons and astrocytes is known as the “glutamate-glutamine cycle” (Figure 2).

**Figure 2.** Schematic representation of the glutamate-glutamine cycle between neurons and astrocytes. Glt: glutamate, Gln: glutamine, GLT-1: glutamate transporter 1, SNAT 3: system N amino acid transporter, SNAT 1-2: system A amino acid transporter 1 and 2
The glutamate-glutamine cycle is the major metabolic pathway of both glutamate and glutamine in the brain. By labeling compounds radioactively it has been shown that the bulk of glutamate transmitter derives from glutamine \(^{67,181}\) and neuronally released glutamate is the substrate in 80-90% of the astrocytic glutamine synthesis \(^{89}\).

However, alternative pathways for glutamate turnover exist. Glutamate can enter the tricarboxylic acid (TCA) cycle in the astrocytes and be oxidatively degraded instead of amidated \(^{113,193}\). Astrocytes can also synthesize glutamate \textit{de novo} by carboxylation of pyruvate, an anaplerosis estimated to account for 10-30% of the exported glutamine \(^{76}\). High extracellular glutamate levels increase astrocytic glutamate oxidation and decrease pyruvate carboxylation \textit{in vitro} \(^{76}\). Thus the overall significance of the glutamate-glutamine cycle in the brain’s total glutamate turnover probably varies.

Reports on interstitial glutamine levels under normal and pathological condition range between 150-1200 μM \(^{88,145,150,202}\). High glutamine levels have been considered neuroprotective \(^{64,174}\) and the GS expression can increase in response to injury and high glutamate levels \(^{139}\). Contrary to most other amino acids there is no significant concentration gradient for glutamine across the blood–brain barrier (BBB) \(^{202}\) but the localization of glutamine transporters at the endothelial–astrocyte interface is such that it favors a net out-flux of glutamine from the brain \(^{73}\).

Eighty percent of the total brain glucose consumption is used to drive the glutamate-glutamine cycle \(^{109,167}\). Neuronal excitation, high extracellular glutamate levels and large astrocytic glutamate uptake are associated with increased astrocytic glucose utilization, primarily through augmented glycolysis \(^{55,107,133,192}\). Glutamate uptake has correspondingly been shown to decrease if the ATP generation is hampered \(^{24}\).

High glutamate levels, low glutamine levels and slow glutamate-glutamine cycling have been described in epileptic brain \(^{136}\) and low glutamine/glutamate ratios, measured with MD, were prognostic of bad outcome in children with TBI \(^{145}\). This indicates that disturbed glutamate-glutamine cycling is of significance in the injured and/or metabolically compromised brain.

The role of glutamate and GLT-1 in the injured brain

Excitotoxicity is a contributing factor to cell damage following acute brain insults \(^{56}\) and elevated interstitial glutamate levels have been reported in TBI \(^{25,123,189,203}\) as well as in SAH \(^{43,53,134,158,162}\), commonly associated with high lactate/pyruvate (L/P) ratios. Several lines of evidence also support the role of excessive neuronal glutamate stimulation in epileptic seizures and epilepsy-induced brain damage \(^{19,31,48,114,115,152,153,189}\).

The role of GLT-1 in the injured brain has received much attention. A transient GLT-1 decrease accompanied by decreased uptake of excitatory
amino acids (EAA) was observed after TBI and decreased GLT-1 levels were associated with aggravated neuronal damage in models of TBI and focal cerebral ischemia. Reversed glutamate transport through GLT-1 has been reported during ischemia and ATP-depletion. Altered levels of GLT-1 were found post-mortem in TBI patients and GLT-1 was absent in cerebral contusions despite high levels of the glia-specific protein glial fibrillary acidic protein (GFAP), which was used as a marker for reactive astrogliosis. These results indicate that astrocytes may decrease their glutamate uptake ability in response to injury.

GLT-1 gene knock-out mice die of seizures and reductions in GLT-1 encoding mRNA was observed in cortex, striatum and hippocampus of genetically epilepsy prone rats. Epileptogenic injections of iron in rat amygdala induced dynamic alterations in GLT-1 mRNA levels. However, there were no alterations in the levels of GLT-1 nor of GLT-1 mRNA in the hippocampus of fully kindled rats.

High GLT-1 activity has been correlated to neuronal survival following ischemic insults. Induction of the GLT-1 expression, which can be obtained pharmacologically by estrogen, β-lactam antibiotics, and glucocorticoids or by sublethal ischemic preconditioning, improves outcome and cell survival in ischemic models. Individuals with a common polymorphism in the GLT-1 promoter region, which results in reduced GLT-1 gene transcription, have higher plasma glutamate levels and worse clinical course following thromboembolic stroke. However, since there is massive glutamate out-flux through the GLT-1 transporter upon dissipation of the membrane potential and energy failure, a high GLT-1 expression may also be harmful and contribute to neuronal death in case of overt ischemia.

Microdialysis

The technique

With MD it is possible to monitor the neurochemical composition of the brain interstitial fluid. A thin (Ø≤1mm) double lumen probe is inserted into the tissue and a physiological salt solution (the perfusate) is slowly pumped through the inlet tubing of the probe. At the dialysis-membrane-equipped tip of the probe the perfusate will equilibrate with the surrounding interstitial tissue fluid by diffusion. The perfusate, now called dialysate, will then contain a proportional concentration of the tissue fluid’s molecules and will pass through an outlet tubing where it can be collected for analysis. To what extent a specific molecule crosses the probe membrane depends on its concentration gradient across the membrane as well as the size of the membrane pores. “Cut-off” is a term defined as the molecular weight at which...
80% of the molecules do not pass the membrane. MD probes with cut-off 20 kDa are the most commonly used clinically. However probes exist in the cutoff range between 6 kDa to 3000 kDa.

Recovery

The ratio between microdialysate concentration and interstitial fluid concentration is defined as the “relative recovery” and reflects to what extent the microdialysate concentration corresponds to the “true” interstitial concentration.\(^{185}\)

\[
\text{Relative Recovery} = \frac{C_{\text{md}}}{C_{\text{ic}}} = 1 - e^{-\frac{K_o A}{F}}
\]

- \(C_{\text{md}}\) = microdialysis concentration
- \(C_{\text{ic}}\) = interstitial concentration
- \(K_o\) = average mass transfer coefficient (cut-off, temperature, diffusion characteristics)
- \(A\) = area of dialysis membrane
- \(F\) = flow rate of perfusate

\[\text{Figure 3. Schematic graphs illustrating how the recovery in microdialysis is dependent on the flow rate of the dialysis fluid and the area of the dialysis membrane}\]

The relative recovery of a substance, probe and flow rate at a specific temperature can be determined \textit{in vitro} by inserting the probe into a fluid with a known concentration and analyzing the microdialysate. One must however be cautious to use \textit{in vitro} recovery rates to calculate interstitial tissue concentrations since the recovery \textit{in vivo} is influenced by additional factors such as rate of diffusion in the interstitial fluid, active transport mechanisms, the
size of the interstitial room and alterations which inevitably will occur with time\textsuperscript{15,16,105}. Methods have been developed to estimate the recovery \textit{in vivo}\textsuperscript{92,116}.

In the no-net flux method\textsuperscript{106} the interstitial concentration of a certain substance is determined by varying the concentration in the perfusate. The perfusate concentration, at which there is no difference in the dialysate and perfusate, i.e. where there is no net flux of the compound across the MD membrane, corresponds to the interstitial fluid concentration.

Another method is the extrapolation-to-zero-flow method where the tissue concentration is derived by varying the flow rate and extrapolating to the dialysate concentration where flow equals zero. Since a minimum amount of dialysate is required for analysis of compounds there is an inverse relationship between relative recovery and time-resolution at which the monitoring can take place.

Performing simultaneous MD in subcutaneous (SC) tissue and analyzing an endogenous reference compound, such as urea, can be used to monitor the performance of the intracerebral probe over time. Stable CNS-urea/SC-urea ratios indicate functioning probes\textsuperscript{151}.

**What and where does microdialysis measure?**

The MD probe tip, inserted in brain tissue, will sample from the interstitial space, which is the fluid filled gap between the cells that normally make up \~20\% of the total brain volume. Several factors, including brain region, depth of insertion, neuronal activity as well as the presence or absence of brain pathology affect the characteristics of this space and consequently will influence the MD measurements\textsuperscript{79}.

Upon probe insertion there will invariably be a certain tissue trauma, which will be reflected in the dialysate. This tissue reaction appears to be transient and most metabolites will return to baseline within 1 hr after the insertion of a continuously perfused probe\textsuperscript{59,78,117}.

It must also be emphasized that although we, by using MD, can measure fluctuations in important brain metabolites, many biomarker signals are typically complex and the technique can not alone answer the questions from where fluctuations in tissue concentration derive\textsuperscript{79}. Interpretation of MD data must consequently be done with caution. Several possibilities are commonly plausible; in the case of glutamate an elevation may be due to neuronal synaptic release, carrier mediated release or overflow from nonsynaptic sources, e.g. cell leakage or BBB breakdown.

**Microdialysis to monitor brain energy metabolism**

MD was introduced as a neurochemical monitoring tool in neurointensive care in 1992\textsuperscript{134}. MD has since been used extensively to monitor the brain’s
energy metabolism since the interstitial levels of glucose, lactate and pyruvate provide information about glucose and oxygen utilization and availability in the tissue. Currently the interstitial L/P ratio is regarded the most sensitive and specific MD marker of ischemia \(^{13}\) and the L/P ratio has been shown to correlate with both the oxygen extraction ratio \(^{53,83}\) and CBF \(^{159}\).

The significance and specificity of different L/P ratio levels is however not established. Normal human MD L/P ratio levels range between 17-27 (based on mean reported values ± 1 SD) \(^{143,165}\). Given this, and the fact that the interstitial L/P ratio is a reflection of the cellular redox state, L/P>40 has been suggested as a threshold value for “tissue energy crises” \(^{188}\). Support for this threshold are observations where L/P>40 occurred in human ischemic brain \(^{81,94}\) and during permanent as well as transient cerebral blood flow reductions in experimental animal models \(^{51}\).

Evidence however exist that cerebral energy crises can be a consequence of other phenomena than reduced blood flow since high L/P ratios have been described in the absence of both ischemia and hypoxia \(^{81,188,191}\). Increasing attention has also recently been devoted to L/P ratio elevations with low pyruvate levels \(^{77}\). These observations indicate that there may be a specific type of energy metabolic crisis that involves impaired glycolytic activity and/or shunting of glucose to other metabolic pathways \(^{12,49}\).
The general aim of this thesis was to study glutamate turnover in acute brain injury. The specific aims were to investigate:

- GLT-1 levels in the iron-induced posttraumatic epilepsy model.

- If reductions in GLT-1 are associated with altered interstitial amino acid levels and to study the effects of a free radical scavenger in the iron-induced posttraumatic epilepsy model.

- Whether energy perturbation is generally associated with low glutamine/glutamate ratios in injured human brain.

- Microdialysis patterns of energy metabolism and glutamate turnover and their relation to clinical events in subarachnoid hemorrhage patients receiving neurointensive care.
Materials and Methods

Animals (Paper I & II)

Male Sprague Dawley rats (BK, Stockholm, Sweden), weighing 200-400 g were used. They were housed 2-3 / cage with free access to food and water. The animals were anaesthetized during all surgical procedures, MD, and at decapitation. Anesthesia was initiated using fluothane in oxygen and maintained with 1-1.5% isoflurane in a 30/70 mix of O₂ and N₂O delivered through a nose cone or via an orotracheal tube. Body temperature was measured rectally and maintained at 37.5 °C using a heating pad. During neuro-surgical and MD procedures the rats were fixated in a stereotaxic frame and the skin to be opened was infiltrated with 1% lidocaine. During MD rats received a catheter in the tail artery through which blood pressure was monitored and blood samples drawn. The rats arterial blood gases remained within the following ranges; pH 7.35-7.45, pCO₂ 4.5-6.9 kPa, pO₂ 11-19 kPa.

Iron-induced posttraumatic epilepsy model

To create the epileptogenic lesion according to Willmore, a craniotomy was made on the left or the right side. Following opening of the dura 5 μl of 100 mM ferrous chloride solution was injected 5 mm laterally of the midline and 2 mm behind bregma 2 mm down the cortex at a rate of <1μl/min using a micropump (CMA 100/ Microdialysis AB, Stockholm, Sweden). The craniotomy was closed with bone-wax and the skin was sutured. Before induction of the lesion, half of the animals in Paper II were intraperitoneally (i.p.) injected with the nitrone radical scavenger α-phenyl-tert-N-butyl nitrone (PBN) (100 mg/kg) (provided by Centaur Pharmaceuticals Inc., Sunnyvale, CA) dissolved in saline. Controls received i.p. saline.

Microdialysis in animals

A MD probe (MAB 6.14.3, Microbiotech, Sweden) with a 15 kDa cut-off, 3 mm long membrane was inserted into the cortex adjacent to the iron-lesion or at the corresponding site in controls. Probes were perfused (2 μl/min) with
artificial cerebrospinal fluid (CSF; Na$^+$ 148 mM, Ca$^{2+}$ 1.2 mM, Mg$^{2+}$ 0.9 mM, K$^+$ 2.7 mM, Cl$^-$ 155 mM) and microdialysate samples (20 μl) were collected in 10-min fractions. The samples were analyzed for amino acids using high performance liquid chromatography (HPLC) with fluorescence detection (CMA/280 Microdialysis AB, Stockholm, Sweden). Twenty primary amino acids were automatically pre-column o-phtaldialdehyde derivatized in a refrigerated CMA/200 autoinjector (Microdialysis AB, Stockholm, Sweden) and gradiently separated on a Nucleosil C18 reverse phase column as described previously 39. Glucose, lactate, and pyruvate were analyzed with enzymatic technique using CMA 600 Microdialysate Analyzer (Stockholm, Sweden). The basal value for each animal was calculated as the mean of three (for amino acids) or two (for metabolites) different fractions collected during the third hour of MD, allowing for wash out of any leakage of metabolites into the interstitial space caused by the insertion of the MD probe. The manufacturer report in vitro relative recovery for this probe and pump rate to be approximately 35% for lactate and pyruvate and 15% for glutamate.

EEG

To study the reproducibility of the model, electroencephalograms (EEG) were done on some of the animals in Paper I in a three-channel montage using subcutaneous stainless needle electrodes and a digital acquisition system (‘Voyageur', Nicolet Inc. Madison, WI). The EEG recordings were analyzed blindly by a co-authoring neurophysiologist without knowledge of whether the animal had been given an injection of ferrous chloride solution or CSF. Transients with an amplitude twice the background amplitude and with a duration of <70 ms were defined as spike potentials, while those with a duration of 70-200 ms were considered as sharp waves.

Immunoblotting

For immunoblotting experiments in Paper I and II, the animals were decapitated, the brains were placed on ice and the tissue around the lesion was removed with a punch (Ø=4 mm), frozen immediately and stored at -75°C. Cell homogenate was prepared from the tissue in lysisbuffer containing protease inhibitors. In Paper I the membrane fraction of the tissue was prepared using washing and ultracentrifugation steps as described previously 140. In Paper II whole cell extract was used. The samples were briefly sonicated and insoluble particles were spun down. 4μg (Paper I) and 12 μg (Paper II) total protein from each animal were prepared with a loading buffer containing sodium dodecyl sulfate (SDS) and β–mercaptoethanol. Electrophoreses was performed on polyacrylamide gels that were transferred to polyvinylidene
difluoride (PVDF) membranes (Bio-Rad laboratories, Hercules, CA). The membranes were incubated with anti-GLT-1 antibodies (Alpha Diagnostic International, San Antonio, Texas) followed by horseradish peroxidase-coupled anti-rabbit secondary antibodies. The protein bands were visualized using enhanced chemoluminiscence (Sigma Aldrich AB, Sweden). Immunoblotting procedures were repeated using antibodies against GFAP (Sigma Aldrich AB, Sweden) (Paper I and II), glutamate and aspartate transport protein (GLAST) (Alpha Diagnostic International, San Antonio, Texas) (Paper I) and β-tubulin III (Biosite, Sweden) (Paper I) and visualized as above. The intensities of the bands were quantified using densiometric scanning and image software.

PBN analysis of blood

Trunk blood was collected from the animals at decapitation and centrifuged at 5000 G for 5 min at 4°C. The supernatant was collected and stored at -70°C. Samples were kept dark at all times. PBN in plasma was analyzed using HPLC as described previously. Lowest detectable level was 2 μg/g. Twenty-four h after the cortical iron injection, at the time of sacrifice/MD; no PBN was detected in blood.

Patients (Paper III-V)

Thirty-three patients (22 women and 11 men) with spontaneous SAH, admitted to Uppsala University Hospital NICU were studied. Mean age was 55.5 (standard deviation, SD 8.9) years. Patients were admitted between April 2003 and September 2005. All were believed to have a potential to survive. The median World Federation of Neurosurgical Surgeons (WFNS) grade was 3.5 (range 1 to 5). The aneurysms were treated surgically (n=16) or with endovascular therapy (n=16). In one patient no aneurysm was found. Demographic data is presented in Table 1. In the neurointensive care protocol, all SAH patients have bed rest and receive nimodipine. The aim is to keep the patients normovolemic and normotensive. Patients with decreased level of consciousness are intubated and artificially ventilated. All intubated patients are sedated with propofol. During the monitoring, all patients had a VD for ICP-monitoring and CSF drainage. We aim at keeping ICP below 20 and normally initiate CSF drainage at 15 mmHg. In case a delayed neurological deficit due to VSP is suspected and new ischemic lesions are absent on CT, the cerebral blood flow is optimized by increasing the blood volume and the blood pressure. The VSP treatment protocol includes maintaining a central venous pressure of 8-10 mmHg and systolic blood pressure above 140 mmHg, 0° head elevation, and administration of dextrane.
Microdialysis in patients

A CMA/70 probe with 10 mm membrane length (CMA Microdialysis, Stockholm, Sweden) and 20 kDa cut-off were placed in frontal (n=30) or temporal (n=3) lobe cortex, either before or during the treatment of the aneurysm, via a separate burr hole in front of the VD (n=28) or through a bone flap (n=5). Care was taken to insert the probe in visually non-injured cortex. The mean duration from first sign of SAH to start of MD was 29.5 (SD 24) h and mean duration of MD sampling was 112 (SD 37) h. CT-scans showed no radiological signs of bleeding around the MD probe in any of the studied patients.

The probes were perfused with artificial CSF at a rate of 0.3 μL/min using a microinjection CMA/106 pump (CMA Microdialysis). Samples were taken hourly and analyzed bedside for lactate, pyruvate, and urea with enzymatic techniques using a CMA/600 Microdialysate Analyzer (CMA Microdialysis). Urea was monitored to control the probe performance. At least 3 h passed after insertion of the probe and start of sampling to allow for normalization of changes owing to probe insertion. The estimated relative recovery using this MD set-up is ~70%.

The CMA/600 Microdialysate Analyzer was automatically calibrated when started as well as every sixth hour using standard calibration solutions from the manufacturer. Quality controls at two different concentrations for each substance were performed every weekday. Imprecision values for between-assay coefficient of variation was <10% for the low and <5% for the high concentration control samples.

Samples were analyzed for glutamate and glutamine with HPLC with fluorescence detection. Briefly, the amino acids were automatically derivatized with o-phthaldialdehyde in a CMA/200 (CMA Microdialysis) refrigerated microsampler and gradient separated on a Nucleosil C18 column 5 μm, 60 mm x 4 mm (CMA Microdialysis). The mobile phase gradient consisted of 0.1 M sodium acetate buffer pH 6.7 with 2.5% tetrahydrofurane and methanol concentration increasing from 5% to 97.5%.

Quality control measurements of the HPLC system using blank water samples, as well as standardized calibration solutions mimicking those of the human samples were run daily. In addition, internal control samples were run together with each patient series. Imprecision values were 3% to 5% for within-assay coefficient of variation and 5% to 9% for between-assay coefficient of variation for glutamate and glutamine.

Computerized multimodality monitoring

A networked computerized multimodality monitoring system at the Uppsala NICU connected to a central server, is used to collect and store physiological patient data such as ICP, mBP, CPP, temperature and SpO2. One value per
minute is displayed and saved for each channel and patient. The data collection is sometimes interrupted when the patient is taken to the operating theatre or during radiological examinations. The data was manually validated and the software was used to identify extreme values. Negative ICP values were either removed or set to zero. In total, 3617 hours at the NICU were monitored with MD and the computerized multimodality monitoring system. For correlation analyses each hourly MD sample measurement was matched to the mean ICP, MAP and CPP during the hour when the sample was collected, taking into account the dead space in the MD tubing which corresponded to a lag phase of 17 minutes. The dialysis fluid in a sample consequently reflected the brain interstitial milieu during the period -77 minutes to -17 minutes from the stamped collection time.

Recording of clinical status and clinical course of events

Patients were grouped depending on WFNS grade at admittance and WFNS grade at the end of monitoring. WFNS grade 1-3 was regarded as good and WFNS grade 4-5 was regarded as bad clinical status. The patients' consciousness level and peripheral neurological status were recorded at 1-hour intervals by the NICU staff throughout the monitoring. Evaluation of the consciousness level was done using Reaction Level Scale 85 (RLS 85). A clinical neurological deterioration was defined as an instant or period during which the patient consciousness level decreased (≥ a one-step increase in the RLS-score) or a new focal neurological deficit appeared. All clinical neurological deteriorations that appeared after initiation of MD were noted. In order to be classified as an event the deterioration should have resulted in an action from the neurosurgeon in charge (e.g. a new CT-scan). Patient charts were retrospectively scrutinized for information regarding CSF drainage, fever-episodes, cardiopulmonary problems, surgical procedures, extubations and radiological examinations. The deteriorations were suspected to be a delayed ischemic neurological deterioration (DIND) unless there were other more likely explanations (e.g. hydrocephalus, fever, hypoxia, and electrolyte disturbance). Brain CT-scans of the patients obtained during and after the monitoring time where retrospectively studied to obtain information about ischemia and bleedings as well as the exact position of the MD probe and its localization to the parent artery of the aneurysm.

Classification of microdialysis patterns

For each patient, the interstitial concentration of lactate, pyruvate, glutamate, glutamine, L/P ratio, and glutamine/glutamate were plotted over time and analyzed for patterns. Periods where the L/P ratio reached above 40, indicating a pattern of “ischemia” and/or “energy crisis” were identified. In Paper III at least two consecutive L/P ratios above 40 were required to be
considered an “energy crises event”. In Paper IV at least five hours with L/P > 40 during a period of 10 hours were required to be classified as an “ischemic pattern”.

In Paper II, the concentration curves for glutamate and glutamine during energy failure periods were examined to determine if interstitial glutamine and glutamate increased or decreased during the period when L/P ratio reached above 40. A “glutamate increase” was when the glutamate concentration was higher in all the “high L/P ratio samples” as compared with the preceding glutamate baseline concentration. In addition, the interstitial glutamate levels should reach above the normal upper reference limit (32 μM) to be classified as an increase. For glutamine, it is currently less clear what to regard as a normal concentration. We defined a “glutamine increase” when the glutamine concentration was higher in all the high L/P ratio samples as compared with the preceding glutamine baseline concentration and/or there was a clear upward slope of the glutamine curve during the high L/P ratio period. A “glutamine decrease” was when the glutamine concentration was lower in all high L/P ratio samples as compared to the preceding glutamine baseline concentration and/or there was a clear downward slope of the glutamine curve during the high L/P ratio period. A pattern where no obvious pattern was discerned, for example in the case where there were both increasing and decreasing interstitial glutamine concentrations during the high L/P ratio period, was classified as “varying”.

In Paper IV all accelerated increases in interstitial glutamine, “glutamine surges”, were identified. A “glutamine surge” was a period of at least 12 h where the interstitial glutamine concentration increased with at least 150 μM per 12 h.

Statistics
Statistical analyses and graphical presentations were performed using Statistica 7.1. for Windows (StatSoft Inc., Tulsa, OK, USA). Results have been regarded significant if $P < 0.05$. Correlation coefficients above 0.20 were considered to be of potential significance. Unless stated otherwise figures in brackets represent mean values ± SD. Parametric and non-parametric statistics on raw and transformed data were used to describe, analyze, and compare MD data.

In Paper I and II, unpaired t-test on raw data was used to compare protein amounts on immunoblots as well as interstitial levels of amino acids and energy metabolites, in different groups.

Raw and logged MD data together with raw pressure data were used for correlation analyses in Paper III and V. Missing data was pair-wise deleted. To test differences in Pearson correlation coefficients, Fisher’s z transformations were conducted.
In Paper III, Wilcoxon matched pairs test was used to compare glutamine levels at start and end of monitoring. Kruskal–Wallis analysis of variance with post hoc testing and multiple comparisons of mean ranks was used to compare glutamine and glutamate during high L/P ratio periods, grouped according to pyruvate level.

In Paper IV, WFNS grade groups were compared with respect to glutamine levels at different time points using unpaired t-test. Fisher’s exact test was used to determine if there was an association between glutamine surges and extubations.

In Paper V, monitoring hours with ICP>10 mmHg were compared to hours with ICP≤10 mmHg using unpaired t-test and Mann-Whitney U-test.

Ethics

The local ethics committee for animal and human research approved all studies. Patients or their relatives had given informed consent to participation.
Results & Discussion

Time dependent protein alterations at the iron-induced epileptogenic lesion.

Both GLT-1 and GLAST are almost exclusively found on astrocytes, with GLT-1 found mainly in cortex and hippocampus, and GLAST found mainly in cerebellum. Paper I was performed in order to investigate GLT-1 and GLAST in the epileptogenic region at different time points following the cortical iron injection. Nine epileptic animals and six control animals were used.

Using immunoblotting techniques the level of GLT-1 (~70 kDa) appeared to be decreased by 38% at day 1 and by 70% at day 5 in the iron-lesioned animals compared to controls (Figure 4). After 3 months, there was no significant difference between the groups, but those results were inconclusive since the variation in GLT-1 levels was great between the animals. GLAST (67 kDa) and β-tubulin III (50 kDa) levels were unchanged at all times after the cortical iron injection.

![Immunoblots](image)

*Figure 4. Immunoblots of control (C) and iron-lesioned (E) animals 5 days after induction of the lesion. Cortex from iron-lesioned animals clearly have decreased intensity of the band corresponding to GLT-1 (~70 kDa), compared with control animals. An extra band (*) was observed at ~45 kDa for GLT-1.*
The amount of GFAP increased with time compared to controls (Figure 5). The progressive and delayed astrocytic reactivity, indicated by GFAP increase at the iron-lesion, may be of importance in the development of epileptogenic lesions. This is supported by studies in patients with posttraumatic seizures, where hemosiderin deposits surrounded by gliosis were identified \(^7,^9,^8\). Gliosis is also a common finding in resected epileptic tissue \(^57\).

![Figure 5](image)

*Figure 5.* GFAP levels at different time-points were compared by measuring the light absorbance using densiometric scanning of the immunoblots. The relative absorbance shows GFAP levels in iron-lesioned animals relative to control animals. Mean relative absorbency (± SD) at day 1, 5 and 90 after induction of the iron-lesion is presented. There was a progressive relative increase of the amount of GFAP in iron-lesioned animals, compared to controls.

**Significance and reproducibility of the animal model**

Bleeding within the cerebral tissue is associated with the development of posttraumatic and post-stroke epilepsy. Extravasation of blood leads to hemolysis and deposition of heme-containing compounds in the tissue. This can initiate a sequence of redox-reactions and free radical generation leading to peroxidation of lipids and proteins resulting in cell death and the formation of an epileptogenic focus \(^199\). Histologically, iron-associated epileptogenic foci in humans and animals are characterized by gliosis, neuronal loss, neuronal deformity and accumulation of iron in macrophage-like cells \(^10,^27,^70,^80,^151\).

All animals that received a cortical iron injection in Paper I displayed epileptiform activity at 3 months, with frequent spike/sharp wave activity (Figure 6). Control rats had no epileptiform EEG activity. This confirms the reproducibility of the model. In other studies more than 90% of the animals present epileptiform activity \(^121,^153\) and epileptogenesis occurred in stages for up to 12 months following the cortical iron injection \(^121\). This resembles hu-
man posttraumatic epilepsy where epileptiform activity usually arise within the first year following the brain injury.  

![ EEG recording from a rat, three months after a cortical iron injection. Frequent spike and sharp wave activity is present. T3 was placed over the left and T4 over the right temporeparietal region. Fz was frontal, in the midline.](image)

There is oxidation-induced aggregation of GLT-1 at the epileptogenic lesion.

In Paper II we extended our investigation of astrocytic glutamate uptake in the posttraumatic iron-induced epilepsy model. The levels of GLT-1 as well as of interstitial amino acids and metabolites were measured using immunoblotting and MD 24 h after a cortical iron injection. Since formation of free radicals is involved in epileptogenesis following stroke and TBI, the effect of a radical scavenger was studied.

Half the animals were given PBN intraperitoneally in a dose that had proven neuroprotective in other brain injury models. Thirty-eight animals were studied and there were four study groups: iron-lesion (F); iron-lesion + PBN (FP); no lesion (C); no lesion + PBN (CP).

There were decreased amounts of GLT-1 monomer (~70 kDa) at the iron-lesion compared to controls. High-molecular weight GLT-1 species were present at the iron-lesion but absent in controls. Iron-lesioned animals pretreated with PBN had more of the 70 kDa band and less of the high-molecular weight bands compared to the iron-lesioned animals not receiving PBN (Figure 7). The total GLT-1 immunoreactivity, the summed intensities of all three bands, was similar in the F group and the FP group. The control groups C and CP had similar GLT-1 immunoreactivity. GFAP did not differ between the four groups, which is in line with the results from Paper I where
increased GFAP immunoreactivity at the iron-lesion appeared at 5 days and 3 months but not at day 1 (Figure 5).

*Figure 7.* GLT-1 immunoreactivity 24 h after a cortical iron injection and the effects of PBN. Immunoblots using antibodies against GLT-1 and GFAP on cell homogenates from iron-lesioned cortex. Each column represents one individual animal. F = homogenate from iron-lesioned cortex, FP = homogenate from iron-lesioned cortex from animals pre-treated with PBN, C = control homogenate from cortex where artificial CSF was injected instead of iron. The GLT-1 70 kDa band was completely absent on one FP animal at the edge of this blot (indicated with •), probably due to technical reasons. Repeat of the immunoblotting procedure for that animal revealed a band pattern similar to the other FP animals, including a 70 kDa band.

Since PBN attenuated the appearance of high molecular weight species of GLT-1, the results indicate that there is oxidation-induced aggregation of GLT-1 at the lesion site. GLT-1 transporters are known to occur in pairs on the cell membrane and steric interaction between two transporters is required for glutamate uptake. In the presence of an oxidant, a GLT-1 pair can bind to each other covalently, impairing their motility. On immunoblots, aggregated transporters will appear as bands with molecular weight about two or three times that of the ~70 kDa monomer. The two larger bands (150 kD and 220 kD) seen in iron-lesioned animals (Figure 7) presumably represent such multimers of GLT-1, induced by the presence of iron in cortex.

Aggregation of GLT-1, as demonstrated in Paper II, is also the most likely explanation behind the decreased levels of GLT-1 monomer reported at day 1 and day 5 in Paper I. However, after having confirmed the GLT-1
antibody specificity on normal brain homogenate, the high molecular weight part of the PVDF membrane was cut off in subsequent Paper I experiments (in order to conserve antibodies). In hindsight this was obviously erroneous and explains why only GLT-1 bands below 140 kDa were detected in in the iron-lesioned animals in Paper I.

Storage of brain tissue can induce oxidation-mediated aggregation of glutamate transporters. The punched-out brain tissue was stored in -75°C for 1-3 months in Paper I and for 6 months in Paper II, before the brain homogenate was prepared. It is possible that the oxidation/aggregation occurred during this storage. However, the striking effects of PBN indicate that aggregation occurred during the first 24 h following the cortical iron injection and argues in favor of this will now be outlined: Pharmacokinetic studies show that peak PBN brain concentration following peripheral administration appear after 30 minutes, PBN has a biological half-life of 134 minutes, and the brain concentration of PBN is similar to, or below, the plasma concentration. We could not detect PBN in plasma 24 h after our i.p. injection. Therefore we assume that no PBN was present in the brain when the tissue was collected and we argue that the major iron-induced oxidation of GLT-1 and attenuation of oxidation by PBN – resulting in the different band patterns in Figure 7 – occurred in vivo and not during storage.

The interstitial glutamine/glutamate ratio is altered at the epileptogenic lesion

Interstitial glutamate concentrations were higher and glutamine concentrations were lower at the iron-lesion as compared to normal tissue (Figure 8). There was a trend, but no statistically significant difference, towards normalized glutamine/glutamate ratios in PBN pre-treated iron-lesioned animals.

GLT-1 mediated glutamate transport is considered the most important mechanism to maintain low interstitial levels of glutamate in cortex. The low glutamine/glutamate ratios at the iron-induced epileptogenic lesions may be a consequence of decreased glial glutamate uptake. Other possible explanations however exist. As outlined above, energy failure with high L/P ratios and collapse of the electrochemical gradient across the cell membrane result in astrocytic glutamate release instead of uptake. The L/P ratios in the iron-lesioned animals (mean 26 ± 6.3) were however similar to the control group C (mean 27 ± 1.7), which indicate that the probe sampled from viable tissue without energy failure. It is possible that the glutamate increase at the lesion represent unspecific leakage through a disrupted BBB, in line with what has been described in human TBI where increased EAA levels were associated with increase in structural amino acids, e.g. threonine. There are steep concentration gradients across the BBB for most amino ac-
ids, with concentrations being far higher in the blood. The reported blood plasma concentration of glutamate and of threonine is approximately 60 times higher than the respective concentration in microdialysate obtained from group C in our study. Both glutamate and threonine were increased in the iron-lesioned brains, but the relative increase in glutamate is more than twice the relative increase in threonine. This suggests a mechanism other than unspecific leakage behind the elevated glutamate at the iron-lesion.

![Graph](image.png)

*Figure 8.* Mean interstitial microdialysis concentrations (± SD) of glutamate, glutamine and the glutamine/glutamate ratio in cortical tissue 24 h after a cortical iron injection. F (n=6, iron-lesion); FP (n=6, iron-lesion + PBN.); C (n=3, no lesion); CP (n=3, no lesion + PBN).

Strong oxidants, such as Fe$^{3+}$, can alter protein conformation, leading to distortion of secondary and tertiary structure, increased aggregation and fragmentation. Such alterations commonly make a protein susceptible to proteolysis and diminution of normal function. It has been shown that lipid oxidation products can impair astrocytic glutamate transport including GLT-1 activity. Oxidative stress further has a role in epileptogenesis and antioxidants have proven to efficiently prevent brain damage and the evolution of epileptiform activity.

We have shown that the major protein responsible for clearance of synaptic glutamate is structurally impaired and that this is associated with elevated interstitial glutamate levels and decreased glutamine levels, in the iron-induced posttraumatic epilepsy model. Our results suggest, on a molecular
basis, one possible epileptogenic mechanism in bleeding-induced epileptogenic lesions.

Human interstitial brain glutamate and glutamine relate to the energy metabolism

Considering the energy demanding cycling of glutamate and glutamine between neurons and astrocytes we hypothesized that the interstitial glutamine/glutamate ratio may be an indicator of the brain’s energy status. In Paper III we examined if energy perturbation, evaluated mainly by the L/P ratio, is associated with low glutamine/glutamate ratios in the brain. MD data from visually non-injured cortex in patients with spontaneous SAH, during their first week of neurointensive care, was used.

We found that glutamate correlate positively with the L/P ratio ($r = 0.68$) and to lactate ($r = 0.65$). The glutamine/glutamate ratio correlated negatively with the L/P ratio ($r = -0.66$). All these relationships were of log–log nature (Figure 9). There was no correlation between interstitial glutamine and L/P ratio and no relation between glutamate and glutamine.

The observed correlation between glutamate and L/P ratio is in line with previous observations $^{135,157,158}$. Noteworthy is also that high glutamate and low glutamine/glutamate ratios predominantly occurred when the L/P ratio exceeded 40 (Figure 9, top), findings that further support L/P ratio 40 as a threshold for “energy crisis” $^{188}$ at which glutamate levels are high $^{135}$.

There was a significant correlation between interstitial levels of glutamine and pyruvate ($r = 0.55$) and this relationship appeared linear (Figure 10A). In 22 out of 33 patients, the glutamine curve and pyruvate curve had similar shapes (Figure 10B).

Since transiently reduced glutamine production associated with energy perturbation could have been missed in the gross correlation analysis where the majority of the MD samples represented normal L/P-ratios with low glutamate levels indicating an efficient astrocytic glutamate uptake, we specifically identified “energy crisis”-periods where L/P ratio exceeded 40. For these periods we determined whether interstitial glutamine and glutamate increased or decreased as compared to the preceding baseline concentration.
Figure 9. Scatterplots of raw (top) and logged (bottom) concentration in MD samples collected from 33 SAH patients at the NICU. (A) Lactate/pyruvate vs glutamate. (B) Lactate/pyruvate vs glutamine/glutamate.

Figure 10. Scatterplot of MD glutamine vs. pyruvate in 33 SAH patients at the NICU. Pearson linear correlation coefficient of raw data in graph is 0.52, $P<0.01$. (B) Glutamine and pyruvate concentrations over time in one patient to exemplify the close relation between the two compounds.
Thirteen periods with “energy crisis” (median duration 20 h, range 2 to 120 h, total duration 404 h) were identified. The median L/P ratio of these 404 samples was 53 (range 40 to 141) and median glutamate level was 110 μM (range 33 to 320 μM). In four out of 13 periods with L/P>40, the interstitial glutamine levels decreased (Figure 11A). In seven out of 13 periods with L/P>40, there was a varying response involving both increasing and decreasing glutamine concentration. In two out of 13 periods where L/P>40, the interstitial glutamine concentration increased, concomitantly to increasing glutamate, lactate, and pyruvate (Figure 11B).

Given the strong correlation between glutamate and L/P ratio it was not surprising to see that high L/P ratio periods always were associated with coincident sharp increases in interstitial glutamate. During overt ischemic conditions it has been shown that extracellular glutamate accumulation mainly is derived from reversed astrocytic glutamate transport due to failing astrocytic membrane potentials. If glutamate leaves the astrocytes, intracellular glutamate concentration will fall and there will be less substrate for GS. A decreased glutamine synthesis would presumably be reflected in the interstitial space since glutamine normally leaves the astrocytes by diffusion.

Figure 11. Examples of MD measurements from two patients to illustrate decreasing vs. increasing interstitial glutamate concentration during an energy crisis period when L/P>40. (A) L/P ratio and glutamate increase sharply at hours 107 to 109. Interstitial glutamine and pyruvate decrease during these 3 h compared with the preceding baseline readings. (B) L/P ratio exceeds 40 during hours 64 to 85. Glutamate, glutamine, and pyruvate increase during this period compared with the preceding baseline readings.
However, when analyzing glutamine during the 13 energy perturbation periods, no definitive pattern emerged; interstitial glutamine concentration sometimes increased and sometimes decreased. Only when pyruvate was considered it was possible to predict whether glutamine and the glutamine/glutamate ratio would increase or decrease during the high L/P ratio period. The relationship between interstitial glutamine and pyruvate, present in the entire material (Figure 10A) as well as in the sub-group of high L/P ratio samples (Figure 12), is a novel observation that may enhance the understanding of the glutamate-glutamine cycle energetics and metabolic dysfunction following brain injury.

Figure 12. Glutamine (Gln) concentration and glutamine/glutamate (Gln/Glt) ratios in MD samples where interstitial L/P>40 (n=404) grouped depending on whether the pyruvate concentration was low (<119 μM, n=214), normal (119-213 μM, n=169) or high (>213 μM, n=21) \(^{143}\). Plots represent median values with the 25%-75% range. The glutamine concentration differed significantly between the three groups \((P<0.01)\). Glutamine/glutamate ratios were lower in the low pyruvate group compared to the normal pyruvate group and the high pyruvate group \((P<0.05)\). The difference in glutamine/glutamate ratio between the high pyruvate group and normal pyruvate group did not reach statistical significance.
L/P ratios above 40 are associated with clinical criteria of ischemia

Patient demographic data, aneurysm localization, description of clinical events and MD patters are outlined in Table 1 and Table 2. In Paper IV an “ischemic MD pattern” was defined as at least 5 hours with L/P>40 during a 10 hour period. There were 12 ischemic MD periods in the material (mean duration 28 h, range 7-120 h).

Six ischemic MD patterns appeared as an L/P ratio increase from a lower baseline concentration (range 10-30 μM) and preceding a DIND (Figure 13, Table 1 and 2). The mean time from the first L/P ratio measurement above 40 to the DIND was 16.7 h (range 5-24 h, SD 8.7 h).

Five patients had ischemic MD pattern at onset of monitoring and there were consequently no baseline measurements. In four out of these five patients, MD was initiated following craniotomy, aneurysm clipping and hematoma-evacuation. The remaining one had the MD probe and a VD inserted due to a rapid neurological deterioration (Table 1 and Table 2). All patients with ischemic MD pattern from monitoring onset developed CT verified infarcts.

In one patient an ischemic MD pattern occurred as an L/P ratio increase from a low baseline during a radiological examination where the anesthesia was complicated. No infarcts were present on subsequent CT-scans of this patient’s brain (Table 1 and Table 2).

These observations indicate that L/P>40 is a severely pathological MD pattern that was associated with functional and/or structural ischemia in 11 out of 12 cases. L/P ratio elevations above 40 from a lower baseline also appear to be an early and specific warning pattern of an upcoming ischemic neurological deterioration since this pattern, 6 out of 7 times, was followed by a DIND.

In our experience MD baseline levels are hard to define, especially during ongoing monitoring. If the L/P ratio is to be used in clinical decision-making there are practical and didactic advantages to use an absolute threshold value instead of a relative increase from baseline. In the present study an absolute threshold of 40 was used. Using this value to predict DINDs there would have been few false positive cases. However L/P ratio measurements above 40 preceded only 40% (6 out of 15) of all DINDs in our material. This low sensitivity may be a consequence of the probe not being close to the ischemic region and/or the fact that L/P>40 presumably only appear in tissue with severely perturbed energy metabolism.
Figure 13. MD patterns in patient #4 who experienced a DIND during monitoring. Arrows indicate DIND onset. The DIND is preceded by an ischemic MD pattern where L/P ratio exceeds 40. Glutamate levels are high. See Table 1 and 2 for more details.

Where was the probe, where should it have been and why?

From Table 1 it is apparent that the relationship between the probe, focal lesions and the vascular territory of arteries varied amongst the patients. This was one major concern when initialising the analysis of the MD patterns and their relationship to clinical events.

In 8 patients the MD probe was localized in a territory supplied by the parent artery of the ruptured aneurysm. In 12 patients the MD probe was localized in a territory that may have been supplied by the parent artery of the ruptured aneurysm. In 12 patients the probe was localized in tissue that was not supplied by the parent artery of the ruptured aneurysm. There were six cases where an ischemic MD pattern preceded a DIND. In four out of these six cases the MD probe sampled from tissue that was not supplied by the affected artery.
In previous attempts to predict DIND using MD, care has commonly been taken to insert the probe in the tissue most likely to be affected by VSP. Skjøth-Rasmussen and co-workers showed that DINDs were preceded by 20% increases in L/P ratio from baseline with a positive predictive value of 85% and peak L/P ratio occurred 4-50 h (mean 23) before DIND onset. We adhered to our routine clinical practice where the most convenient time for probe insertion commonly is in conjunction with emergency ventriculostomy most often without a priori knowledge of the aneurysm localization. Nevertheless, in 6 out of 7 (86%) cases where L/P ratio increased above 40 from a low baseline this was followed by a DIND. On average, the first measurement of L/P>40 occurred 16.7 h (range 5-24 h) prior to the DIND. This yields a specificity and a time-window similar to that reported by Skjøth-Rasmussen et al. Noteworthy is also that in 4 out of 6 DINDs that were preceded by an ischemic MD pattern in our cohort, the probe was outside the territory supplied by the aneurysmatic artery. This indicates that perhaps little is gained by individualizing the MD probe placement in SAH patients and a more standardized, global, placement of the probe may be simpler and yield equally specific information.

Table 1. (see page 43) Patient demographics, aneurysm localization, treatment regimes, MD information, occurrence of DIND and associated radiological findings and MD patterns in the 33 studied SAH patients. PC = posterior circulation, ICA = internal carotid artery, MCA = medial cerebral artery, ACA = anterior communicating artery, E = embolization, C = clipping, VD = with ventricular drain, BF = via bone flap, R.fro = right frontal lobe, L.fro = left frontal lobe, R.tem = right temporal lobe, L.tem = left temporal lobe, Y = yes, N = no, M = maybe, D = glutamine surge occurs during DIND, A = glutamine surge occurs after DIND, I = new ischemic lesion on CT, H = new hemorrhagic lesion on CT, 0 = no new lesion on CT, V = patient received VSP treatment, LP = L/P>40 occurs as an increase from baseline preceding a DIND, LP* = L/P>40 occurs from the beginning of monitoring, LP** = L/P>40 occurs as an increase from a lower baseline during a complicated anesthesia and not associated with a DIND.
<table>
<thead>
<tr>
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Table 2. Description of the 15 DINDs that occurred in the 33 patients, DIND-associated MD patterns, CT results and initiated VSP treatment. LP = L/P > 40 occurs as an increase from baseline preceding a DIND, LP** = L/P > 40 occurs as an increase from a lower baseline during a complicated anesthesia and not associated with a DIND. D = a glutamine surge occurs during the DIND, A = a glutamine surge occurs after DIND, I = new ischemic lesion on CT, H = new hemorrhagic lesion on CT, 0 = no new lesion on CT. V = patient received VSP treatment.

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Interstital glutamine increase over time and glutamine surges may signal hard-working astrocytes

In 28 out of 33 patients, glutamine levels were higher at the end of sampling compared with the beginning of the sampling and there was an overall significant increase in glutamine during the monitoring (Figure 14). Glutamine increases often came in sharp surges and this pattern was intriguing. In Paper IV and V one important aim was therefore to determine if there were clinical events associated with these glutamine surges.
Figure 14. The median interstitial glutamine concentration during the first 6 hours of sampling (start) and during the last 6 sampling hours (end) for each of the 33 patients. The difference between each start and end value is also presented. Glutamine concentrations increased during the monitoring time ($P<0.001$).

DINDs are associated with glutamine surges

Twelve out of 15 DINDs were associated with a glutamine surge (Table 1 and 2). In eight patients, the glutamine surge came during the deterioration and in four patients the glutamine surge came immediately following the DIND (Figure 15). Structural ischemia was absent on CT in seven DIND patients and these patients received VSP treatment. In five VSP-treated patients the only DIND-associated MD pattern was a glutamine surge.

Neurons release glutamate at a lower ischemic threshold than astrocytes and neuronal glutamate release accelerate astrocytic glutamate uptake, glycolysis and ATP utilization. We therefore suggest that the observed increase in glutamine and pyruvate during DIND is a favorable response in metabolically challenged tissue where the astrocytes maximize their glycolytic rate to yield ATP for glutamate uptake. Increasing glutamine and pyruvate levels in patients with suspected VSP, without ischemic MD-pattern and without ischemic CT signs, may consequently indicate increased neuronal glutamate release where the astrocytes increase the glutamate-glutamine cycle turnover by augmented glycolysis. As long as the astrocytes can accelerate the glutamate uptake the only clue that there is a high glutamate-glutamine cycle turnover will be increasing interstitial glutamine levels. The theory that astrocytes, by their relative higher resistance to oxygen and glucose deprivation, can rescue endangered neurons during ischemia, is supported by experimental data.
Glutamine surges appear when ICP is lowered.

Three out of our 33 patients had high mean hourly ICP-readings, in the 15-25 mmHg range, and their clinical deterioration or lack of improvement was partially attributed to the high ICP. The L/P ratio and glutamate were relatively normal during the period when ICP was high in these patients. To lower the ICP, CSF drainage at 10 mmHg was initiated. The lowering of ICP down to 10 mmHg was associated with sharply increasing glutamine and pyruvate levels in these three patients (Figure 16). We interpret this MD pattern as a sign of energy metabolic boost upon alleviation of a constraint that, in these three cases, was an unfavorably high ICP. One interpretation of these three cases is that moderately increased mean hourly ICP (15-25 mmHg) can hamper the brain tissue energy metabolism. This observation also generates a question whether SAH patients with moderately elevated ICP (15-20 mmHg) generally will benefit from CSF drainage at a lower pressure level than what is stated in current clinical treatment protocols.
Figure 16. Intracranial pressure (ICP), cerebral perfusion pressure (CPP), pyruvate and glutamine in one out of three patients where a glutamine surge was associated with ICP lowering achieved by increased CSF drainage. This patient underwent embolization of a left posterior cerebral artery aneurysm at monitoring hour 16 after which he was severely obtunded but obeyed command when pain stimulated. He had progressing right sided arm weakness from monitoring hour 38 and onwards. A CT-scan revealed suspected new ischemia in the territory of the left posterior cerebral artery. The ventricular drain was opened at 10 mmHg at monitoring hour 83 (arrow). As ICP decreased to 10 mmHg there was a surge in interstitial Gln and pyruvate.

Interstitial glutamine relate to the clinical admittance status

Patients with poor WFNS grade at admittance (WFNS 4-5, n=16) had significantly lower glutamine at the onset of monitoring as compared to good WFNS grade patients (WFNS 1-3, n=17) (Figure 17). Glutamine levels 48 h after the SAH onset were higher in good WFNS grade patients (mean 486±174 μM, n=14) than in poor WFNS grade patients (mean 379±140μM, n=17) but this difference was not statistically significant (P=0.07). Mean glutamine levels during the monitoring was not significantly different (P=0.10) between the group with good recovery (WFNS 1-3 at the end of monitoring) as compared to those with worse recovery (WFNS 4-5 at the end of the monitoring time).
Figure 17. Glutamine levels at onset of microdialysis monitoring in good WFNS grade (1-3) patients (n=17) and poor WFNS grade (4-5) patients (n=16). Glutamine levels at monitoring onset were lower in poor WFNS grade patients than in good WFNS patients (P=0.03). SE = standard error of the mean.

Cessation of sedation does not explain the glutamine surges

Cessation of sedation and extubation were carried out in twelve out of the 33 patients during the monitoring. There were 23 glutamine surges in the entire material. Ten patients had both an extubation and a glutamine surge during the monitoring. In six of these ten cases the glutamine surge started within a time window of -12 h and +12 h from the time point of extubation. Overall, there was no association between presence of extubation during the monitoring and presence of a glutamine surge during the monitoring (P=0.46).

Intracranial hemodynamics and microdialysis measurements

In Paper V the overall relationship between brain energy metabolism, the glutamate-glutamine cycle activity and intracranial hemodynamics was studied. Correlation coefficients between MD variables and pressure parameters are presented in Table 3.
Table 3. Pearson linear correlation coefficients for hourly microdialysis measurements and pressure variables in 33 SAH patients receiving neurointensive care. Significant ($P<0.05$) correlation coefficients above 0.20 are presented.

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</tr>
<tr>
<td>pyruvate</td>
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<td>0.24</td>
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<tr>
<td>L/P</td>
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<tr>
<td>glutamate</td>
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</table>

Glutamine and pyruvate correlated positively with CPP and these variables increased over time and during the NICU stay (Figure 14 and Figure 18). It is possible that an increasing interstitial concentration of pyruvate and glutamine, which was observed in the majority of the patients, is a consequence of improved cerebral perfusion. The relationship between CPP, MAP and pyruvate also provides additional indication, on a brain tissue metabolism level, that very high CPP may be beneficial following SAH 156. There was a negative correlation between glutamine and ICP (Figure 18C) and an even stronger correlation between glutamine/glutamate and ICP (Table 3). These observations are in line with the notion that low interstitial glutamine/glutamate ratios are associated with brain tissue constraint 145. MD patterns were generally better when ICP was below 10 mmHg as compared to hours when ICP exceeded 10 mmHg (Table 4). Previously it has been shown that SAH patients experiencing ICP above 20 mmHg have higher interstitial glutamate and L/P ratio levels as compared to patients without high ICP periods 161. High ICP (>20 mmHg) periods are also associated with clinical deterioration following SAH 156.

Table 4. Mean interstitial concentration during hours when ICP<10 mmHg (n=780) and during hours when ICP>10 mmHg (n=2723) in 33 SAH patients. For all metabolites the difference between the groups is statistically significant.

<table>
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<td>pyruvate µM</td>
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<td>142</td>
<td>&lt;0.01</td>
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</table>
Figure 18. Scatterplots of hourly recordings in 33 SAH patients receiving neurointensive care, (A) cerebral perfusion pressure vs. glutamine, (B) cerebral perfusion pressure vs. pyruvate, (C) intracranial pressure vs. glutamine.
Interstitial glutamine – what does it mean?

The glutamate-glutamine cycle is the major cerebral metabolic pathway for both glutamate and glutamine. One important observation in this patient material was that fluctuations in glutamine levels over time were slower and less pronounced than for glutamate. This may not be surprising considering the high capacity systems for both glutamate release and glutamate uptake. Glutamine, on the other hand, travels more slowly, with both release into and uptake from the interstitial space partly depending on concentration gradients across the cell membranes of neurons and astrocytes. The absolute level of interstitial glutamine at any given time is therefore presumably a consequence of the astrocytic production rate, the rate of astrocytic release and the rate of neuronal uptake, during the preceding period.

Data on normal human brain interstitial glutamine levels is scarce. In pediatric TBI patients and in adult NICU patients where MD set-ups similar to ours were used, the highest glutamine level was 572 and 1200 μM, respectively. In animals, the reported basal brain interstitial glutamine concentration range between 150 and 1000 μM. In view of this, glutamine levels above 1000 μM, which were present in a proportion of our observations, are elevated. Ability to produce glutamine has been associated with neuronal survival when brain cells are subjected to stress and injured brain tissue with high glutamate levels up-regulate GS. Such up-regulation may be one explanation behind the observed glutamine increase over time that occurred in the majority of the patients (Figure 14).

Reduced GS activity has been associated with increased interstitial glutamate levels despite normal amounts of EAAT-2. The very low glutamine levels early after SAH may be a sign of an overall low glutamate-glutamine cycling due to global ischemia associated with the bleed, with suppressed astrocyte energy metabolism and little glutamine synthesis due to lack of substrate and/or energy. Decreased glutamine uptake from blood could also be an explanation behind the low initial glutamine levels, since the energy requiring uptake from blood has been shown to decrease during ischemia.

Up to this point glutamine elevations in the patients have been explained by increased glutamate-glutamine cycle activity. However, alternative mechanisms behind the interstitial glutamine increases deserve consideration. One possible glutamine source is flow from the blood across the BBB. However, the glutamine transport from plasma to the brain interstitial space is normally highly regulated. It occurs in both directions but commonly with a net out-flux from the brain. In previous work and in work underway we have seen little correlation between alterations in plasma levels and brain interstitial levels for various amino acids, including glutamine and glutamate. In addition, critical illness such as SAH is associated with decreasing plasma glutamine levels. This argues against that the observed fluc-
tuations in brain interstitial glutamine levels are significantly influenced by extracerebral sources.

Neurons depend on glutamine uptake for restoration of the presynaptic storage of vesicular transmitter glutamate. Decreased neuronal glutamine uptake would theoretically cause increased interstitial glutamine levels. However experimental data on SNAT 1-2 activity indicate that neuronal glutamine uptake is unaffected or even augmented in brain injury models.

When classifying the MD pattern a “glutamine surge” was defined as at least 150 μM increase in interstitial concentration over at least 12 h. Most often the increase during a surge was much greater than that. Twenty-three glutamine surges were present in the material. Retrospectively it was possible to identify 15 clinical events, either a DIND (n=12) and/or a substantial decrease in ICP (n=3), that coincided with a glutamine surge. For the remaining glutamine surges it was not possible to undoubtedly point out a corresponding event when examining the charts, write-ups and the digitally stored physiological data. However there is a range of possible influencing factors that were not taken into account in this study, e.g. minor alterations in CSF drainage levels and volumes, propofol and morphine requirements/administration and improvements/deteriorations in consciousness level that were not picked up by the blunt RLS 85 grading.

One obvious hypothesis was that patients wakening up were the cause of the increasing glutamine levels. However neither was there an association between cessation of sedation/extubation and glutamine surges, nor was there a difference in glutamine levels at monitoring end (data not shown) or mean glutamine levels during the monitoring (Paper IV), between patients who woke up and those who did not.

Notably, the highest glutamine levels (>2 500 μM, Figure 11B, hour 133) in our material was not found in the metabolically most “normal” brain but in a brain with signs of intense energy production as well as a high interstitial glutamate load. This leaves us with a belief that the intriguing glutamine increase over time in a majority of the patients presumably represents hypermetabolism in astrocytes that have been subjected to stress, including neuronal glutamate release.

Origin of low glutamine/glutamate ratios in animals and humans

It is difficult to directly compare MD concentrations of metabolites obtained in animals and humans, mainly due to differences in probe membrane length, perfusion rate and the overall recovery. However, the glutamine/glutamate ratio and the L/P ratio are presumably recovery-independent.
The glutamate and glutamine levels measured in Paper II are in line with other works where 3 mm MD membrane probes and a perfusion rate of 2 μl/min were used\textsuperscript{118,187}. The glutamine/glutamate ratios in the iron-lesioned animals were always below 3.3 (see Paper II and Figure 8). In the human series only 3% of the monitored hours had glutamine/glutamate ratios below 3.4 and this was associated with overt ischemic L/P ratios (mean L/P ratio was 49 when glutamine/glutamate ratio was below 3.4). Iron-lesioned animals with very low glutamine/glutamate ratios had mean L/P ratio below 30. This, together with the immunoblotting data, support that it is a structural impairment of GLT-1, and not energy failure, that is responsible for the elevated glutamate levels and the decreased glutamine levels at the iron-lesion.

Methodological and statistical considerations

MD patterns are highly individualistic\textsuperscript{122} and dependent on a range of factors\textsuperscript{79} why interpretation of real-time MD data is difficult at the bedside. In this thesis an attempt was made to determine how MD patterns relate to clinical events. An approach where MD data is visually observed, classified and correlated to clinical events, is difficult, time consuming and to some extent subjective. Nevertheless semi-quantitative observations of dynamic MD patterns and their relation to clinical course may be necessary in order to pursue beyond the existing knowledge that patients with overall abnormal MD measurements generally are worse off.

Many MD variables are not normally distributed and this appears to be especially true for glutamate where the brain has powerful mechanisms to maintain low interstitial levels. Once these mechanisms fail to work there are exponential increases in the interstitial concentration due to additive, pathological processes. Currently no consensus exists on how to treat MD data in statistical analyses and it is often at the discretion of the author. Throughout this thesis parametric and non-parametric statistics have been used on raw as well as on transformed data.

When exploring potential relationships in MD patterns, correlation analyses were done using all monitoring hours from all patients. There are problems with such a practice since each of the 3600 monitoring hours was treated as an independent measure. This is not true, since a number of measurements are repeated and many measurements come from the same patient. It must also be considered that the hemodynamic parameters, to some extent, were controlled variables since intervention (e.g. CSF drainage) was initiated once they exceeded certain limits. These limitation and violations need to be considered when evaluating the presented results.

To summarize, this thesis is partly based on data from patients receiving intensive and individualized care. Observational and descriptive analyses were performed. This inherently involves a range of limitations. Neverthe-
less, *in-vivo* measurements from injured human brains have been presented together with careful analyses of clinical parameters. Hopefully these findings will lead to better questions, deeper excursions and ultimately improved understanding of pathophysiological and recovering processes in the injured brain. Furthermore, the combination of MD and advanced neuroimaging (e.g. magnetic resonance spectroscopy and positron emission tomography), not readily available in this study may further potentiate the process of improved understanding of the neurochemistry and pathophysiology of acute brain injury.
Concluding remarks

This thesis spans over animal experiments and clinical observations on glutamate turnover in the injured brain. The major findings are:

Astrocytic glutamate uptake protein GLT-1 is structurally impaired and aggregated in iron-induced epileptogenic cortical lesions in rat, probably as a consequence of oxidative damage.

Reduced monomeric GLT-1 levels are associated with increased interstitial glutamate levels and decreased interstitial glutamine levels at the iron-lesion in rat. These amino acid alterations are most likely a consequence of decreased astrocytic glutamate uptake and may play a role in the development of posttraumatic epilepsy.

In human SAH, the brain interstitial glutamine/glutamate ratio correlates inversely with the L/P ratio. Glutamine increase over time and there is a correlation between glutamine and pyruvate. During periods of energy crises, defined as L/P ratio above 40, glutamine will increase or decrease depending on whether pyruvate is high or low.

Low interstitial pyruvate levels may signal a reduced energy producing capacity, unrelated to ischemia, which hampers the energy demanding glutamate-glutamine cycling.

Interstitial L/P ratios above 40 following SAH is a pattern associated with functional and/or structural ischemia. Delayed ischemic neurological deficits are associated with L/P ratio above 40 and/or surges in interstitial glutamine. Surges in interstitial glutamine predominately occur during delayed ischemic deteriorations where high L/P ratios and radiological ischemia are absent.

Cerebral perfusion pressure correlates with interstitial pyruvate and glutamine. Intracranial pressure correlates with glutamine and the glutamine/glutamate ratio. Glutamine surges appeared when a high ICP was substantially lowered by increased CSF drainage.
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