N-acetylaspartate in brain

– studies on efflux and function

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A doctoral thesis at a university in Sweden is produced either as a monograph or a collection of papers. In the latter case, the introductory part constitutes the formal thesis which summarizes the accompanying papers. These have already been published or are in a manuscript at various stages (in press, submitted or in manuscript).

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Still confused…on a higher level

ABSTRACT

N-acetylaspartate (NAA) is an amino acid derivative present in high concentration in the brain. The function of NAA is still unsettled in spite of 50 years of research. The mainly neuronal synthesis and glial breakdown of NAA requires a well regulated neuronal efflux and glial uptake. In the present work hippocampal slices were used to study how NAA efflux from neurons is regulated and to further investigate possible functions of NAA.

For the determination of NAA a reversed phase HPLC method with UV detection was developed. The method allowed for the simultaneous determination of NAA and creatine and was comparable or better in sensitivity than previous methods based on UV detection.

A newly developed efflux protocol that allowed the determination of efflux and delayed cell death was used to study NAA efflux in cultured hippocampal slices. Activation of the NMDA receptor, a glutamate-receptor subtype that is involved in learning and memory but also in nerve cell death following stroke, evoked a prolonged Ca^{2+}-dependent NAA efflux from cultured slices. The efflux of NAA was not due to unselective membrane rupture but at high NMDA concentrations the efflux of NAA correlated with the NMDA-mediated *delayed* (24 hours after efflux) excitotoxicity. However, no causal relationship between delayed excitotoxicity and extracellular NAA could be demonstrated as culturing with high concentrations of NAA was non-toxic.

Extracellular osmolarity was decreased moderately for 10-48 hours to address the proposed function of NAA as an osmoregulator but no change in the tissue content of NAA was observed from either cultured or acutely prepared hippocampal slices. However, depolarisation resulted in efflux of NAA from acutely prepared slices that could be reduced both by a NMDA-receptor blocker and hyperosmotic solution.

Culturing of hippocampal slices with the monomethyl ester of NAA increased intracellular NAA levels. This was followed by reduced levels of the anion phosphoethanolamine and a tendency towards decreased Cl^- concentration in the slices. NMDA-mediated delayed excitotoxicity was unaffected by increased intracellular NAA concentration.

Overall, the results suggest that the NMDA receptor is involved in the regulation of NAA efflux from neurons. Increased extracellular as well as intracellular NAA is non-toxic and NAA does not seem to function as an important Ca^{2+} chelator or as an osmoregulator under physiological decreases in osmolarity.

LIST OF PUBLICATIONS

This thesis is based on the following published articles or manuscripts, which are referred to in the text by their roman numerals.

I. Mattias Tranberg, Malin H. Stridh, Barbro Jilderos, Stephen G. Weber and Mats Sandberg
Reversed phase HPLC with UV-detection for the determination of N-acetylaspartate and creatine
Analytical Biochemistry (2005) Aug; 343(1):179-82

II. Mattias Tranberg, Malin H. Stridh, Yifat Guy, Barbro Jilderos, Holger Wigström, Stephen G. Weber and Mats Sandberg
NMDA-receptor mediated efflux of N-acetylaspartate: physiological and/or pathological importance?
Neurochemistry International (2004) Dec; 45(8):1195-204

III. Mattias Tranberg, Abdul-Karim Abbas and Mats Sandberg
***In vitro* studies on the efflux of N-acetylaspartate by changed extra- and intracellular osmolarity**
Submitted

IV. Mattias Tranberg and Mats Sandberg
N-acetylaspartate monomethyl ester increases N-acetylaspartate concentration in cultured rat hippocampal slices: effects on excitotoxicity and levels of amino acids and chloride
Submitted

Related work not included in the thesis:

Camilla Wallin, Abdul-Karim Abbas, Mattias Tranberg, Stephen G Weber, Holger Wigström and Mats Sandberg
Searching for mechanisms of N-methyl-D-aspartate-induced glutathione efflux in organotypic hippocampal cultures
Neurochemical Research. (2003) Feb; 28(2):281-91

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LIST OF ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ANAT	acetyl-N-aspartate transferase
ASPA	aspartoacylase
BDNF	brain derived neurotropic factor
CaCC	Ca^{2+}-dependent Cl^--channel
CFS	cerebrospinal fluid
CNS	central nervous system
GABA	γ-aminobutyric acid
HPLC	high performance liquid chromatography
MRS	magnetic resonance spectroscopy
NAA	N-acetylaspartate
NAAG	N-acetylaspartylglutamate
NAA MME	N-acetylaspartate monomethyl ester
NaC3	Na^+-coupled carboxylate transporter 3
NADH	reduced nicotinamide adenine dinucleotide
NMDA	N-methyl-D-aspartate
PEA	phosphoethanolamine
PNS	peripheral nervous system
PI	propidium iodide

INTRODUCTION

The brain is unarguably the most remarkable organ in the body. Although much knowledge about the brain has been obtained during the last century there are still many unsolved questions. One of the neurochemical mysteries is the high concentration of the amino acid derivative N-acetylaspartate (NAA) in brain. The functional role of NAA is still unsettled despite its discovery 50 years ago.

Cells of the nervous system

The central nervous system (CNS) contains two main types of cells; the nerve cells (neurons) and the glial cells (glia). The conduction of electrical signals occurs through processes that originate from the nerve cell body. Branches receiving signals are called dendrites and the transmitting processes are termed axons. The axon terminates in synapses, the contact zones between nerve cells. One nerve cell can have up to 100 000 synapses on its dendritic tree.

The glial (Greek for glue) cells are divided in microglia and macroglia. The microglia are normally found in a resting state but are activated by infection, injury or disease and may be involved in neural repair mechanisms. The macroglia cells are in turn divided into three main categories; astrocytes, oligodendrocytes and Schwann cells. The astrocytes are responsible for upholding an appropriate chemical environment around the neurons. Oligodendrocytes (found in the CNS) and Schwann cells (found in the peripheral nervous system, PNS) produce the myelin sheet, a fatty layer isolating the axon which improves signal conduction. An additional non-neuronal cell type that is found in the lining of the ventricles is the ependymal cell.

Glutamatergic neurotransmission

The most common form of synapse in the adult vertebrate CNS relies on release of a chemical compound (neurotransmitter) for transmission of signals between cells. Neurotransmitters can be inhibitory, excitatory or modulatory depending on the neurotransmitter and the receiving receptor types. The most common excitatory neurotransmitter in the vertebrate brain is glutamate.

Glutamate binds to three main types of receptors; the N-methyl-D-aspartate (NMDA)-, the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate/kainate (AMPA/kainate or non-NMDA) and the metabotropic glutamate-receptors. The NMDA and AMPA/kainate type of receptors are ligand-gated cation-channels. In a typical glutamatergic synapse released glutamate causes depolarisation via the influx of Na^+ through AMPA/kainate-receptors. The depolarisation may remove a Mg^{2+} ion bound to the receptor channel of the NMDA receptor which allows for influx of both Na^+ and Ca^{2+}. The influx of Ca^{2+} is important since Ca^{2+} is a second messenger affecting a number of enzymatic systems that can modulate the post-

synaptic terminal and its future response to glutamate, a process termed synaptic plasticity (Fig. 1). The depolarization of the post-synaptic terminal creates a passive current that travels the dendrite towards the cell body and contributes to the generation of a new action potential.

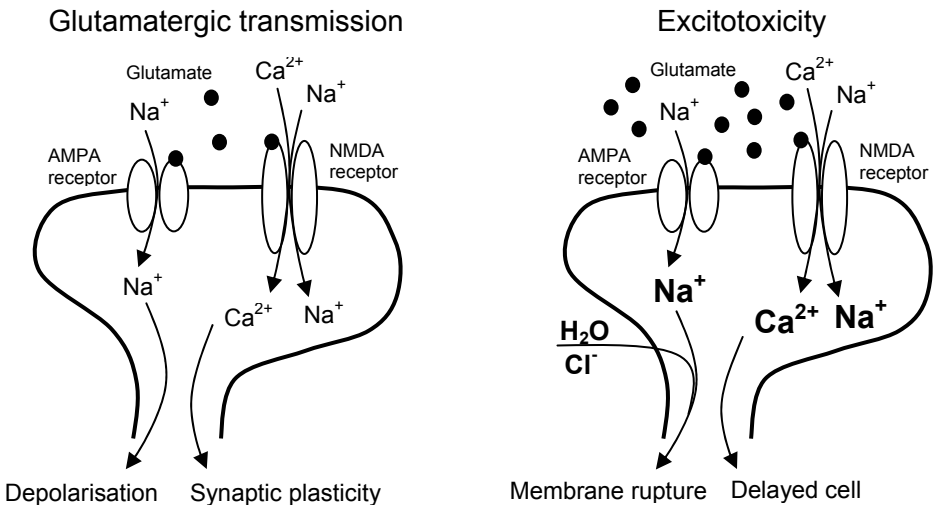

Figure 1. Normal glutamatergic transmission occurs by release of glutamate from the pre-synaptic terminal (not shown) and activation of AMPA and NMDA receptors resulting in influx of Na^+ and Ca^{2+} in the dendrite. Excessive concentrations of glutamate overactivate the glutamate receptors which lead to excessive influx of Na^+ and Ca^{2+}. This can result in excitotoxicity by immediate membrane rupture or delayed cell death.

Excitotoxicity

Overactivation of NMDA and AMPA receptors causes a large influx of cations (Na^+ and Ca^{2+}) to the cell which can result in acute and/or delayed cell death, termed excitotoxicity (Martin et al., 1998) (Fig. 1). Acute excitotoxicity is characterized by membrane rupture and is caused by water and Cl^- influx that follow the cation influx. The mechanisms underlying the delayed cell death are not fully understood but the influx of Ca^{2+} through the NMDA receptor is an important initiating factor (Sattler and Tymianski, 2000). Elevated intracellular Ca^{2+} increases the production of free radicals and activates different types of enzymes such as phospholipases, proteases and endonucleases (Irving et al., 1996; Phillis and O'Regan, 2004). An uncontrolled elevation in intracellular Ca^{2+} may therefore overactivate these pathways leading to oxidative stress, breakdown of the cytoskeleton and degradation of DNA which eventually leads to cell death (Sattler and Tymianski, 2000; Atlante et al., 2001). Excitotoxicity is one mechanism of nerve cell death that occurs for example in stroke and may be important also in chronic degenerative diseases such as Parkinson's and Alzheimer's diseases.

N-ACETYLASPARTATE

Chemistry

NAA is an amino acid derivative with an acetyl group on the amine group (Fig. 2). NAA thus has two negative charges at physiological pH.

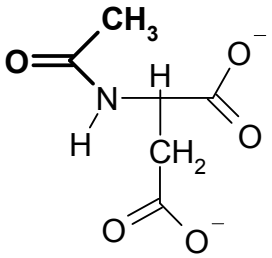

Figure 2. The chemical structure of NAA at physiological pH with the acetyl group shown in bold.

Determination

Chromatographic techniques

Determination of NAA was initially carried out by separation of NAA by anion exchange chromatography, hydrolysis and the subsequent determination of aspartate by the ninhydrin method (Moore and Stein, 1954; Tallan, 1956).

Various methods for HPLC based determination of NAA has been developed throughout the years. The most frequently used was developed by Koller and co-workers 1984 and utilises anion exchange chromatography and UV-detection (Koller et al., 1984). HPLC methods using ion-pairing with UV detection as well as reversed phase methods using mass spectroscopy and fluorescence for detection has also been described (Korf et al., 1991; Ma et al., 1999; Tavazzi et al., 2000).

Gas chromatography with hydrogen flame ionisation detection was relatively early used for the determination of NAA in brain extracts and urine but the use of gas chromatography for NAA determination today is relatively scarce (Marcucci and Mussini, 1966; Miyake et al., 1981).

Enzymatic techniques

The enzymatic method for determination of NAA is based on the hydrolysis of NAA by aspartoacylase. This can be an alternative in laboratories where chromatographic methods are unavailable. The liberated aspartate is converted to oxaloacetate which in turn is metabolised to malate in a NADH-dependent manner. The decrease in NADH can then be measured spectrophotometrically at

340 nm. The method has been used for determination of NAA in brain extracts (Jacobson, 1959; Fleming and Lowry, 1966).

Magnetic resonance spectroscopy

Due to its high concentration NAA has one of the strongest signals in magnetic resonance spectroscopy (MRS). However, it is necessary to use high field strength to resolve NAA from other acetylated compounds such as N-acetylaspartylglutamate (NAAG). The benefit of MRS is its ability to measure NAA levels *in vivo* but MRS has also been employed for determination of NAA in brain extracts (Bothwell et al., 2001; Thatcher et al., 2002). In MRS studies the NAA content is often expressed as a ratio to the assumed stable creatine level. This has been questioned and absolute concentrations, by use of for example external standards, should preferentially be used (Li et al., 2003).

Localisation and distribution

NAA was first discovered in cat and rat brain in 1956 by Tallan (Tallan, 1956). Low or undetectable amounts were found in kidney, liver, muscle and urine (Tallan, 1956; Koller et al., 1984). The brain tissue of mammals and birds contain the highest levels of NAA (up to 10 mM) while the brains of amphibians, invertebrates and reptiles contain low or no NAA. Fishes have intermediate concentration (up to 5 mM) (Birken and Oldendorf, 1989; Burri et al., 1990). Furthermore NAA is present in the PNS and the retina at concentrations about 5-10 fold less than the levels in the CNS (Nadler and Cooper, 1972a; Miyake and Kakimoto, 1981; Ory-Lavollee et al., 1987). More recently the presence of NAA in the lens of rat, hog and different fish species has been observed (Baslow and Yamada, 1997).

Regional and developmental distribution in the CNS

Higher levels of NAA in the cortex compared to the brain stem have been reported in several studies (Fleming and Lowry, 1966; Miyake and Kakimoto, 1981; Koller et al., 1984). A more uniform distribution has, however, also been described (Blakely et al., 1987). The developmental increase in NAA is well established (Tallan, 1957; Miyake and Kakimoto, 1981; Koller and Coyle, 1984; Burri et al., 1990; Florian et al., 1996; Tkac et al., 2003). The increase is relatively uniform in the different regions of the CNS and also occurs in white matter areas and the PNS (Nadler and Cooper, 1972a; Koller and Coyle, 1984; Florian et al., 1996; Tkac et al., 2003). An exception appears to be the eye which has a constant concentration of NAA during development (Florian et al., 1996). In humans an increase in NAA in the striatum from about 5 to 9 mM was found from week 30 of gestation to about 1 year of age (Toft et al., 1994).

Cellular and extracellular distribution

From the early studies of Nadler and Cooper it was suggested that NAA was a mainly neuronal compound with a small fraction existing in the oligodendrocytes (Nadler and Cooper, 1972a). Immunohistochemical studies and the finding that NAA levels were reduced after decortication and kainate injection in the striatum and the hippocampus supported this idea (Koller et al., 1984; Moffett et al., 1991; Simmons et al., 1991). NAA was also found in primary cultures of cortical and cerebellar neurons. Unexpectedly the highest concentration was demonstrated in primary cultures of O2A-progenitor cells, a bipolar oligodendrocyte precursor cell (Urenjak et al., 1992). Immature oligodendrocytes also contained NAA while no NAA was detected in mature oligodendrocytes, type-1 astrocytes or meningeal cells (Urenjak et al., 1992). However, a different culturing protocol induced differentiation of the O2A-progentitors and synthesis of NAA in mature oligodendrocytes (Bhakoo and Pearce, 2000). Contrary to these findings Baslow reported the absence (< 0.1 mM) of NAA in cultured cerebellar granule cells, cortical neurons, astrocytes, oligodendrocytes as well as O2A-progenitors (Baslow et al., 2003). In addition, no developmental increase of NAA in cultured organotypic brain slices was reported (Baslow et al., 2003). NAA has also been found in neurons from the PNS (dorsal root ganglia neurons) while it was absent in Schwann cells and perineural fibroblasts (Bhakoo et al., 1996).

Intracellularly, NAA is localised to mitochondria and the cytosol but not to the nucleus or the microsomal fraction (McIntosh and Cooper, 1965; Reichelt and Fonnum, 1969; Petroff et al., 1992). In white matter such as the optic nerve NAA is mainly located in axons (Bjartmar et al., 2002).

The concentration of NAA is low in human urine and plasma (Matalon et al., 1988; Jakobs et al., 1991). In lumbar cerebrospinal fluid (CSF) NAA was found to be about 2 μM with increasing concentration in subarchnoid CSF and about 20-40 μM in ventricular CSF (Swahn, 1990; Sager et al., 1995; Faull et al., 1999). Using microdialysis, the estimated extracellular level of NAA in the cortex, hippocampus and striatum of rats ranges from 23 to 105 μM (Taylor et al., 1994; Lin et al., 1995; Sager et al., 1997; Sager et al., 1999b). The mean dialysate concentration of NAA in a group of patients with traumatic brain injury was 145 μM (Belli et al., 2006).

Metabolism

Synthesis

NAA is synthesized by the enzyme acetyl-N-aspartate transferase (ANAT) (Fig. 3). The first reports of brain NAA synthesis were published in 1959 by the independent work of Jacobson and Goldstein (Goldstein, 1959; Jacobson, 1959). The synthesis of NAA was initially shown to be slow (Reichelt and Kvamme, 1967; Nadler and Cooper, 1972b; Mukherji and Sloviter, 1973). This has later been

confirmed using the MRS technique. Infusion of labelled glucose in rats for 200 min only labelled 3-4% of the total NAA in cerebellum, cortex or hippocampus while aspartate showed a fractional enrichment of 20-25% in all three regions (Tyson and Sutherland, 1998). In patients with Canavan disease, who have elevated NAA levels, the NAA synthesis rate is even slower suggesting substrate inhibition of ANAT (Moreno et al., 2001). The turnover of NAA under normoxic resting conditions in rat is 2-3 days (Choi and Gruetter, 2004). No synthesis of NAA has been observed in non-nervous tissue (Benuck and D'Adamo, 1968; Truckenmiller et al., 1985).

In an important study Patel and Clark showed that NAA is synthesised in rat brain mitochondria in an oxygen and ADP-dependent manner. The synthesis increased with age, in line with the developmental increase in NAA concentration (Patel and Clark, 1979). The K_m values for aspartic acid and acetyl-CoA are about 0.75 and 0.075 mM respectively (Madhavarao et al., 2003; Lu et al., 2004).

NAA can also be produced from the breakdown of NAAG (see page 14).

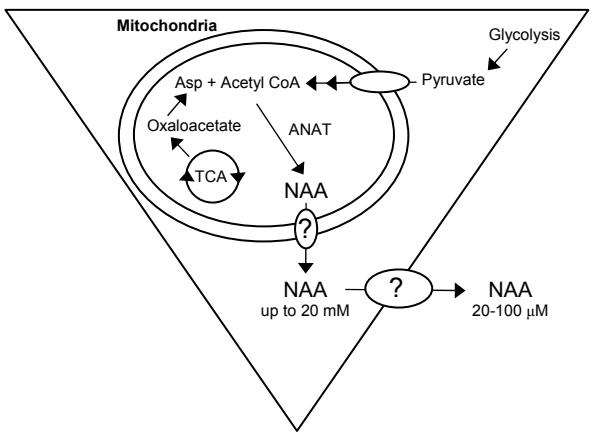

Figure 3. Schematic picture of neuronal NAA synthesis in the mitochondria. NAA is transported to the cytoplasm and the extracellular space by unknown mechanisms. TCA, tricarboxylic acid cycle.

Breakdown

Acetylated amino acids are hydrolysed by a group of enzymes termed acylases or amidohydrolases. The first report regarding a specific acylase, aspartoacylase (ASPA, EC 3.5.1.15), for hydrolysis of NAA was published 1952 (Birnbaum et al., 1952). In contrast to ANAT, ASPA show high activity outside the CNS with expression in the kidney, heart, liver, skeletal muscle and the mammary gland. NAA can be metabolised to CO_2 or used for lipid synthesis in these organs (Berlinguet and Laliberte, 1966; Benuck and D'Adamo, 1968; D'Adamo et al.,

1973). Intracerebrally injected NAA is also rapidly degraded with a 80% reduction in 60 minutes (Nadler and Cooper, 1972b).

ASPA activity increases during development with an almost 10-fold increase in rat brain from birth to adulthood (D'Adamo et al., 1973). The most marked increase occur in white matter regions such as the optic nerve, brain stem and corpus calossum while little activity is observed in the hippocampus and cortex (Bhakoo et al., 2001; Kirmani et al., 2003).

ASPA was partially purified and characterised in the late 1970ies. The enzyme has a mainly cytosolic localisation, a pH optimum around 8 and a K_m of 0.5 mM for NAA (Goldstein, 1976; D'Adamo et al., 1977; Le Coq et al., 2006). The enzyme has highest activity towards NAA but also hydrolyses other acetylated amino acids (Goldstein, 1976; D'Adamo et al., 1977). A deficiency in ASPA was shown in Canavan disease (see page 16), which stimulated research on purification and cloning of ASPA from bovine brain (Matalon et al., 1988; Kaul et al., 1991; Kaul et al., 1993). The protein consists of 313 amino acids and has a molecular weight of 36 kD (Kaul et al., 1993). The gene consists of 6 exons and five introns and is located on chromosome 17 in the 17p13-ter region (Kaul et al., 1994). ASPA also show conservation throughout evolution and an ASPA-like enzyme has been found in a prokaryote (Hess, 1997). ASPA has a similar sequence as the Zn-peptidase superfamily, a group of enzymes that cleave the C-terminal amino acid residue in proteins and peptides (Makarova and Grishin, 1999). Posttranslational glycosylation is also essential for proper ASPA function (Le Coq et al., 2006).

The cellular localisation has been studied using cell culture and histochemical techniques. ASPA is present in primary cultures of O2A-progenitors, immature and mature oligodendrocytes and type-1 and 2 astrocytes (Baslow et al., 1999; Bhakoo et al., 2001). The presence of ASPA in purified myelin is debated (Chakraborty et al., 2001; Klugmann et al., 2003; Madhavarao et al., 2004). Histochemical and immunohistochemical studies confirmed the primary localisation of ASPA in oligodendrocytes but a few microglia and neurons were also stained (Kirmani et al., 2003; Madhavarao et al., 2004). ASPA has also been shown to be present in several cell types of the eye including neuronal cells such as the retinal ganglion cells and photoreceptor cells (George et al., 2004).

Transport

Transport of NAA across the blood brain barrier does not occur (Berlinguet and Laliberte, 1966). In the brain, NAA can be taken up by astrocytes by the Na^+-coupled carboxylate transporter NaC3 (Sager et al., 1999a; Fujita et al., 2005). NaC3 show little activity in neurons (Yodoya et al., 2006). The transport of NAA into astrocytes was shown to be electrogenic with co-transport of Na^+ and NAA in a 3:1 ratio and a K_m for NAA of about 100 μM (Sager et al., 1999a; Fujita et al.,

2005). The transporter is also present in the kidney and liver, the two major regions of ASPA localisation outside the brain (Huang et al., 2000).

Proposed functions

Myelin precursor

In two related papers D'Adamo and co-workers first reported on incorporation of the acetyl moiety of NAA into myelin. The incorporation was highest in 8 and 16 days rats (D'Adamo and Yatsu, 1966; D'Adamo et al., 1968). Incorporation of NAA into brain lipids was more efficient than free acetate and incorporation increased up to 22 days of age (Burri et al., 1991). Transport of NAA from the axon and incorporation of NAA-derived acetate into myelin was shown in the optic system (Chakraborty et al., 2001). Myelin lipids and acetate are also reduced in an ASPA-deficient mouse, further indicating a role for NAA in myelin synthesis and maintenance (Madhavarao et al., 2005). The increase in ASPA during development correlates well with the time of myelination (D'Adamo and Yatsu, 1966; Kirmani et al., 2003).

N-acetylaspartylglutamate precursor

A structurally related compound to NAA is the dipeptide NAAG. It is the most abundant neuropeptide in the brain and is involved in neuromodulation via NMDA and metabotropic glutamate receptors (Neale et al., 2000). The hydrolytic enzyme, glutamate carboxypeptidase II, is present mainly on the surface of astrocytes and generates NAA and glutamate upon hydrolysis (Berger et al., 1999). The synthesis of NAAG by a non-ribosomal enzymatic process has been shown to occur in astrocytes (Gehl et al., 2004). A signalling system between neurons and glia that is based on the release of NAA from neurons followed by synthesis of NAAG in glia has been proposed (Baslow, 2000).

Osmoregulator/molecular water pump

Tissue NAA was shown to be decreased by hyponatremia suggesting a role of NAA in osmoregulation (Sterns et al., 1993). This finding was later supported by microdialysis studies showing increased dialysate levels of NAA induced by hypotonic solution (Taylor et al., 1995; Davies et al., 1998). NAA was also decreased in brain slices after acute hypoosmotic shock (Bothwell et al., 2001). The release of NAA *after* depolarisation with high K^+ has also been suggested to occur as a response to the swelling of neurons (Taylor et al., 1994).

Due to its metabolic compartmentalisation, NAA has been suggested to be a molecular water pump removing excess water produced by neuronal energy metabolism (Baslow, 2002).

Neurotransmitter/neuromodulator

When applied to the crayfish stretch-receptor nerve no stimulatory or inhibitor effect of NAA at concentrations from 10 μM to 1 mM could be detected (Jacobson, 1959). In addition no excitatory or inhibitory effect was found when NAA was applied ionophoretically to spinal neurones (Curtis and Watkins, 1960). After these initial experiments NAA was thought not to participate in neurotransmission. However, using the stellate ganglion of the squid, it was concluded that NAA at high extracellular concentration (20 mM) may serve a neuromodulatory role by increasing neurotransmitter release (Cecchi et al., 1978).
More recently it was shown that intracerbroventricularly injected NAA induced absence-like and convulsive seizures. Also neurons in the CA3 area of the hippocampus were depolarised by NAA and this could be blocked by a broad-range glutamate-receptor antagonist (Akimitsu et al., 2000). Application of NAA to dissociated hippocampal neurons resulted in an inward current due to activation of metabotropic glutamate receptors (Yan et al., 2003). In line with the putative function of NAA as a neuromodulator is the finding that NAA can increase cAMP levels in homogenates of cerebral cortex (Burgal et al., 1982). Obviously, further studies are necessary to settle if NAA has a role in neurotransmission and neuromodulation.

Balancing the anion deficit

The idea that NAA serves to balance the apparent anion deficit in neurons was suggested already in 1957 (Tallan, 1957). This idea was supported by its slow metabolism (McIntosh and Cooper, 1965). The anion deficit is based on calculations of common intracellular anions compared to cations which results in a deficit in anions of about 91 mM to maintain electroneutrality (McIntosh and Cooper, 1965). Part of this deficit is made up by negatively charged proteins and lipids but with its high intracellular concentration and double negative charge NAA makes a large contribution.

Modulating Ca^{2+} levels

NAA in aqueous solution has been shown to chelate Ca^{2+} with a binding constant somewhere between that of the intracellular proteins calsequestrin and calmodulin (Rubin et al., 1995a). In line with these data, rat brain slices from cortex show a 20% decrease in basal uptake of $^{45}Ca^{2+}$ when incubated with concentrations of NAA of 1.25-5 mM (Berdichevsky et al., 1983). Also, extracellular NAA (2-20 mM) reduces the increase in intracellular Ca^{2+} in stimulated astrocytes (Rael et al., 2004). However, it has also been shown that NAA at high concentrations (>3 mM) increases intracellular Ca^{2+} in NTera2-neurons in a NMDA-receptor dependent manner (Rubin et al., 1995b).

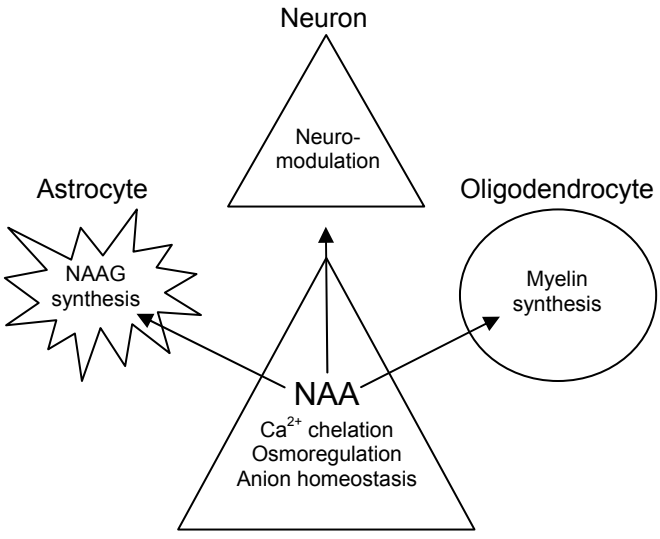

Figure 4. Schematic picture of the proposed functions of NAA.

Pathological and physiological variations in NAA concentration

The early research on changes in NAA concentration in different pathological states or by treatment with different chemicals has been summarized by Birken and Oldendorf (1989). The use of MRS has now generated a vast amount of literature describing changes in NAA levels in human brain in a variety of physiological and pathological situations. In general a decrease in tissue NAA level is found in association with brain disease (Tsai and Coyle, 1995) NAA levels are shown to be decreased after stroke, in Alzheimer's disease, in AIDS, in amyotrophic lateral sclerosis, in Huntington's disease, in multiple sclerosis, in epilepsy and a number of other brain pathologies (Tsai and Coyle, 1995). In recent years the use of MRS for studying cognition has yielded interesting data on NAA. For example white matter NAA has been shown to positively correlate with intelligence and with better cognitive performance (Jung et al., 1999; Ferguson et al., 2002).

Two remarkable exceptions regarding variations in NAA levels need to be mentioned. A pathological elevation of NAA exists in Canavan disease. This is due to a mutation in the ASPA gene resulting in high extracellular NAA levels (Kaul et al., 1993). Canavan disease is a progressive leukodystrophy which usually results in death in early childhood. Spongy degeneration of the white matter is a characteristic feature (Matalon et al., 1995). A single report on the absence of NAA in human brain has also been published. A 3-year old boy showed neurodevelopmental retardation and moderately delayed myelination in combination with no detectable NAA (Martin et al., 2001). However, both the

reported absence of NAA and the neurological deficits have been questioned (Arnold et al., 2001; Sullivan et al., 2001).

AIMS

The overall aim of this thesis was to study how the efflux of NAA from neurons is regulated and to investigate the function of NAA in brain. The specific aims were:

- Develop a HPLC method for NAA determination
- Characterise the NAA content in cultured hippocampal slices
- Study efflux of NAA in relation to delayed excitotoxicity
- Study efflux of NAA in response to changes in osmolarity
- Develop a tool to modify NAA levels
- Investigate the effect on increased intracellular NAA on anion content and delayed excitotoxicity

METHODS

Cultured hippocampal slices

Hippocampal slices were cultured by the interface method described by Stoppini and co-workers (Stoppini et al., 1991). In short Sprague Dawley rats of 8-9 days age were decapitated and the hippocampi were rapidly removed. Each hippocampus was cut in 400 μm thick slices with a McIlwain tissue chopper. Groups of 4 slices were transferred to Millicell membranes (Millipore CM; Bedford, MA, USA) and placed in a six-well plate with 1.3 ml culture medium. Slices were cultured for 12-14 days at 36 °C in 95% air and 5% CO_2. Culture medium (1.2 ml) was changed twice a week. The culture medium included Basal medium Eagle (50%), Earl's basal salt solution (25%), horse serum (23%), penicillin/streptomycin (25 U/ml), L-glutamine (1 mM) and D-glucose (7.5 g/L).

Comments:

In contrast to acutely prepared slices, slice cultures can be kept viable with intact cytoarchitecture for weeks (Bahr, 1995; Gahwiler et al., 1997). Hippocampal slice cultures are thus an *in vitro* model suitable for long-term studies of for example delayed neurotoxicity. Some differences compared to *in vivo* tissue have been documented. One finding that may be of high relevance for the levels of NAA in slice cultures is that the immature isoform of lactate dehydrogenase is present still after for 4 weeks of culturing, indicating an incomplete shift to aerobic metabolism (Schousboe et al., 1993). The high concentration of glucose (42 mM) in the culture medium may also favour anaerobic metabolism and glycolysis and also results in a relatively high osmolarity compared to the calculated osmolarity of the artificial cerebrospinal fluid (ACSF) used in efflux experiments. However, in pilot experiments we found no difference concerning NMDA-mediated efflux in normal ACSF or in ACSF with 30 mM sucrose added to increase osmolarity.

Acutely prepared hippocampal slices

Acutely prepared hippocampal slices were obtained from Sprague Dawley rats of 28-38 days of age. The rats were anaesthetised with isoflurane, decapitated and the brain was dissected out on a cold surface and put in ice-cold ACSF. The hippocampi were removed and 400 μm thick slices were prepared using a McIllwain tissue chopper or a vibratome. Slices were put in oxygenated ACSF (95% O_2 and 5% CO_2) and left to recover for 1 to 3 hours at room temperature before efflux experiments.

Comments:

In contrast to cultured slices acutely prepared slices can be obtained from animals of any age. A disadvantage is that viability of acutely prepared slices in ACSF is relatively short. The tissue deconstruction that occurs by preparation results in necrosis at the cut edges of the slice. This may in turn lead to NMDA receptor

activation, free radical formation and cell swelling in the remaining healthy part of the slice. To reduce these effects slices were always prepared in ice-cold ACSF and left to recover for at least 1 hour in oxygenated ACSF at room temperature before experiments. The current procedure of slice preparation has been used extensively in our lab for preparation of slices for electrophysiological recordings (Dozmorov et al., 2003).

Efflux protocol for cultured hippocampal slices

Slices were incubated in serum free culture medium for 30 min followed by ACSF for another 30 minutes before efflux experiments (Fig. 5). After a final wash with ACSF, the membranes were moved to a new six-well plate which was put in a water bath (34 °C). The atmosphere inside the plate was kept at 60% O_2, 35% N_2 and 5% CO_2 by directing a flow of gas into a water filled container inside the plate and performing the incubation with the lid on. The efflux experiments were carried out by incubating the slices with ACSF (400 µl) on top of the membrane for 5 min. The fluid was then removed and filtered before immediate HPLC analysis or storage in -20 °C (maximally two weeks). This incubation procedure was repeated 9 times (totally 45 min) with most modifications of the ACSF occurring during the fifth incubation period (20-25 min) (Fig. 5).

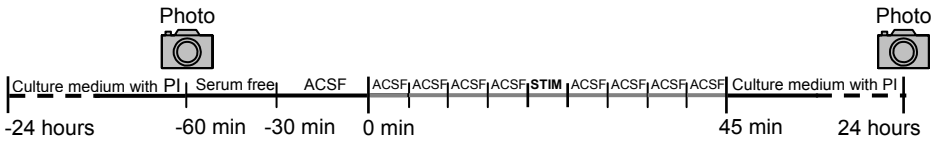

Figure 5. Time scale of efflux experiments with cultured hippocampal slices. PI, propidium iodide (see page 21)

Comments:

This efflux protocol allows for the determination of changes in extracellular neurochemistry that occur immediately after initiation of excitotoxic insult. The development of cell death can then be measured using propidium iodide (PI) (see page 21). The use of the interface method for culturing slices results in a layer of glia cells between the membrane and the major body of the slice. This may slow down the diffusion of substances through the membrane (Schultz-Suchting and Wolburg, 1994). We therefore incubated the slices with ACSF added on top of the membrane in a similar way as described earlier for acutely prepared and cultured slices (Vornov et al., 1998; Wallin et al., 2003). Incubation of cultured slices in 60% instead of 95% or 20% O_2 has been suggested to be more physiological (Pomper et al., 2001). Since, in our efflux experiments, incubation with 95% O_2/5% CO_2 was occasionally accompanied by elevated spontaneous efflux of taurine, phosphoethanolamine (PEA) and glutathione we chose incubation in 60% O_2/5% CO_2. The culturing medium contains high concentrations of amino acids.

Therefore a washing step with serum-free culture medium followed by ACSF was performed before efflux experiments to reduce the background concentration of amino acids.

Efflux protocol for acutely prepared hippocampal slices

Three slices were placed in a 5 ml container with a sealed bottom. The slices were immobilized at the bottom of the container with a small net mounted on a platinum ring. The container with the slices was put in an ACSF-containing well in a 12-well culture plate and a lid was placed on the container. The plate was placed in a water bath (34 °C). The ACSF surrounding the container was continuously gassed with 95% O_2 and 5% CO_2 and the gas flow was led inside the container through small holes in the upper part of the container wall to keep the incubation fluid oxygenated and at the appropriate pH. Incubation fluid was added and removed using syringes and tubing passing trough the lid. All solutions were equilibrated with a gas-mixture of 95% O_2 and 5% CO_2 before use. Efflux experiments were carried out by incubating the slices in ACSF (400 µl) for 5 min similarly to the procedure above for hippocampal slice cultures. The incubation procedure was repeated 14 times (totally 70 min) with modifications of the ACSF during the eight or eight and ninth incubation period (35-40 min or 35-45 min).

Comments:
Acutely prepared slices are generally maintained at 95% O_2 and 5% CO_2. In electrophysiological recordings using a similar submerged type of incubation, sufficient oxygen supply is often maintained by using a high flow-rate of pre-gassed incubation fluid. In our static incubation-protocol a continuous flow of 95% O_2 and 5% CO_2 over the incubation fluid in combination with oxygenation of all solutions prior to use assured sufficient oxygenation and correct pH. In addition, basal efflux of glutamate was low in controls indicating that the slices are kept viable during the whole incubation period. Static incubation-protocols has successfully been used earlier for efflux experiments in our lab and for electrophysiological experiments (Koerner and Cotman, 1983; Li et al., 1996).

Determination of cell death in cultured hippocampal slices

Propidium iodide (PI; final concentration 2 µM) was added to the culture medium at 12-24 hours before photographs were taken with a digital camera (Olympus DP50) coupled to an inverted fluorescence microscope (Olympus IX70) equipped with a rhodamine filter. Photographs were captured using Studio Lite and View Finder Lite softwares (Pixera Corporation, Los Gatos, USA).

For efflux experiments photographs of the slices were then taken before and after incubation (Fig. 5). Then the CA1, CA3 and background areas were encircled and the fluorescence intensity of each area was measured by Scion Image software after conversion to grey scale (Scion Corporation, Frederick, MI, USA). The

fluorescence intensities obtained in slices before the efflux experiments were subtracted before calculation. The fluorescence intensity 24 hours after adding 300 μM NMDA to the culture medium was used as a value of maximal nerve cell death (Vornov et al., 1998). The fluorescence intensity in incubated slices above that of controls (i.e. non-incubated slices), was then expressed as the percentage of maximum fluorescence intensity according to the following formula:

$$Fluorescence\ intensity\ of\ PI\ (\%\ of\ max) = \frac{(Incubated - Control)}{(Max - Control)} \times 100$$

where
Incubated = the fluorescence intensity in incubated slices 24 hours after the efflux experiments,
Max = fluorescence intensity of slices subjected to 300 μM NMDA for 24 hours,
Control = fluorescence intensity of non-incubated control slices 24 hours after the efflux experiments.

Comments:
The use of PI as a marker for cell death correlates well with other methods of cell death determination and it is possible to measure cell death in distinct regions of the hippocampal slice (Noraberg et al., 1999). Exposure time was always adjusted to the fluorescence intensity of slices subjected to 300 μM NMDA for 24 hours (Max) in order to avoid overexposure. The expression of cell death in relation to a "Max" value instead of in absolute values reduces the inter-experiment variations associated with the fluorescence measurements (for example by changes in lamp intensity and differences between PI solutions)

HPLC determination of NAA and creatine

NAA and creatine were separated at room temperature on a TSK-GEL ODS-80T_M column (250 x 4.6 mm; 5 μm particle size; Tosoh, Tokyo, Japan). The mobile phase consisted of 50 mM NaH_2PO_4 (pH 2.15) and was sparged with N_2 before use. A flow rate of 1 ml/min was used and detection was carried out by absorbance at 210 nm. After each sample set (typically 20-40 injections) the column was washed with 20% and 100% methanol (Rathburn, Walkersburn, UK) for 40 minutes each.

Comments:
The method for NAA and creatine determination is discussed separately on page 30.

HPLC determination of glutathione and amino acids

Glutathione and amino acids were determined using *o*-phthaldialdehyde derivatization and fluorescence detection essentially as described earlier (Lindroth

and Mopper, 1979; Sandberg et al., 1986). A solution of β-mercaptoethanol, Na_2-EDTA and NaN_3 (final concentration 20, 1, 5 mM respectively) was added to the samples and standards to keep glutathione in its reduced form and prevent bacterial growth. The amino acid derivatives were separated on a Nucleosil C_{18} column (200 x 4.6 mm; Macherey-Nagel, Germany) with a mobile phase consisting of NaH_2PO_4 (50 mM, pH 5.28) and methanol in a gradient from 25-95% methanol. A flow rate of 1 ml/min was used. Detection was carried out by excitation at 333 nm and emission over 418 nm.

Comments:
HPLC is a highly efficient method for separating and determining substances, for example amino acids, in complex salt containing mixtures such as brain homogenates and ACSF used in the present work. The use of *o*-phthaldialdehyde to label primary amines prior to separation allows the use of fluorescence for detection. This greatly enhances sensitivity compared to non-fluorescent techniques. The method does not discriminate between oxidised and reduced glutathione due to the use of β-mercaptoethanol in the reagent solution. However, in earlier studies from our lab it has been shown that the main part of the total released glutathione after NMDA is in the reduced form (Wallin et al., 1999).

Increasing NAA levels by N-acetylaspartate monomethyl ester

N-acetylaspartate monomethyl ester (NAA MME) was used to increase NAA levels in cultured hippocampal slices. Culture medium with appropriate reduction in osmolarity was prepared by reducing NaCl in the Earl's basal salt solution. Addition of a neutralised stock solution of NAA MME resulted in isoosmotic "loading medium". Slices were cultured for 10-11 days before the switch to "loading medium". Slices were analysed for intracellular content after 3-5 days of culturing in "loading medium".

Comments:
Esterified compounds are commonly used for transferring charged compounds into cells. An example is the acetoxymethyl esters of the Ca^{2+} chelators BAPTA and Fura which are broken down inside the cell by non-specific esterases generating acetic acid and formaldehyde as by-products. The use of NAA MME to increase NAA levels is discussed separately on page 36 in this thesis.

Cl^- determination

Cl^- was determined by a colorimetric method utilising the displacement of SCN^- from $Hg(SCN)_2$ by Cl^- and the subsequent reaction of SCN^- with Fe^{3+} to form the coloured $Fe(SCN)^{2+}$ (Murphy, 1987). In brief, samples were mixed with 0.25 M $Fe(NO_3)_3$ in 9M HNO_3 in a 96-well plate and saturated $Hg(SCN)_2$ in ethanol was immediately added. After 10 min the absorbance was read at 460 nm (SpectraMAX

PLUS, Molecular Devices, Sunnyvale, CA, USA). NaCl in 0.3 M $HClO_4$ was used as standard.

Comments:
Non organic ions are frequently measured by atomic-absorption. This technique was not available to us and we therefore chose a colorimetric method as an alternative. The measurement of Cl^- was not affected by high concentrations of NAA, gluconate or NAA MME. Standard curves of Cl^- made in H_2O, $HClO_4$ or ACSF were not different indicating that the assay was not affected by other common inorganic anions.

Protein determination

Protein determination was carried out according to Lowry or Whitaker and Granum (Lowry et al., 1951; Whitaker and Granum, 1980). Samples were dissolved in 2 M NaOH before analysis. Bovine serum albumin was used as standard.

Statistics

All data were expressed as means ± SEM and p values <0.05 were considered statistically significant. ANOVA with Tukeys or Dunnets post hoc tests were used when several values were compared to each other or to a common control respectively. When two values were compared unpaired one- or two-tailed t-test was used.

SUMMARY OF RESULTS AND ADDITIONAL DATA

HPLC determination of NAA (Paper I)

A reversed phase HPLC method with UV detection was developed for the simultaneous determination of NAA and creatine. The purity of the NAA and creatine peaks in samples was verified by different approaches; calculation of recovery after standard addition to samples, analysing the absorbance profiles of the NAA and creatine peaks (Paper I, Fig. 2) and comparison of the NAA concentration with values determined using the method of Koller and co-workers (Koller et al., 1984). NAA and creatine was separated from a large number of metabolites by the current method (Paper I, Fig. 1). The method was linear from 45 pmol to at least 18 nmol injected (0.5 to 200 μM) for NAA and 9 pmol to at least 3.6 nmol injected (0.1 μM to 40 μM) for creatine.

NAA, glutathione and amino acid content in cultured hippocampal slices *in vivo* (Paper I)

When hippocampal slices were cultured for three weeks no increase in NAA concentration was observed (Paper I, Table I and Fig. 6 in this section). The taurine, PEA and glutathione concentration followed the developmental profile of the hippocampus *in vivo* but the taurine and PEA levels were lower in cultured slices (Fig. 6). It can be noted that the glutamate level was decreased in cultured slices at all time points compared to the hippocampus developed *in vivo* (average decrease of 44 %) whereas that of GABA decreased first after two weeks of culturing (Fig. 6). The most dramatic difference in concentration between cultured slices and hippocampus developed *in vivo* was the concentration of hypotaurine which had an average concentration of 91 nmol/mg protein (average increase of 2090 %) in cultured slices (Fig. 6).

To get an indication of the cellular localisation of NAA in the cultured slices 300 μM NMDA was applied for 24 h in order to induce neuronal cell death. This resulted in a reduction of NAA, glutamate and GABA by 87, 57 and 83% respectively. Other amino acids and glutathione were not affected by NMDA application (Fig. 6).

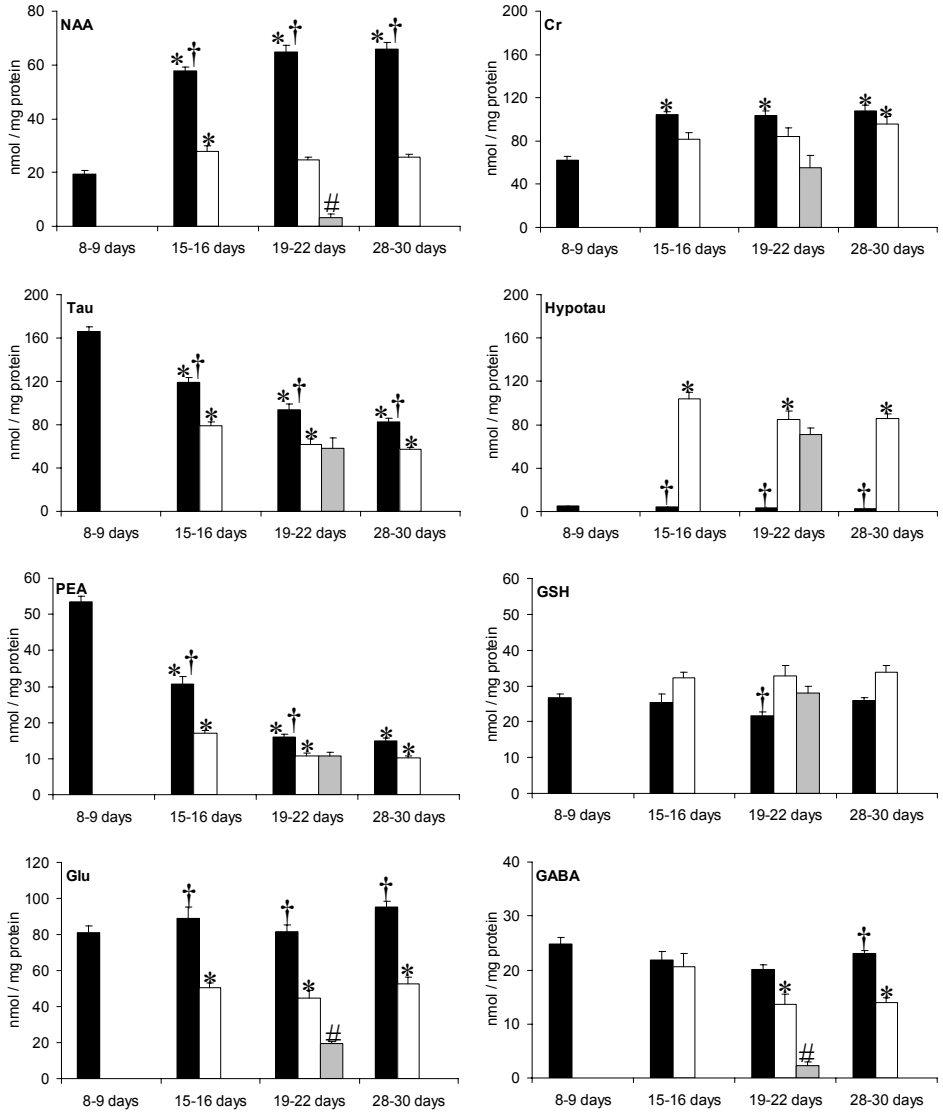

Figure 6. Concentrations of NAA, creatine (Cr), glutathione (GSH) and amino acids in the hippocampus developed *in vivo* (black bars, n = 6) and hippocampal slice cultures (white bars, n = 12-14) from rat. x-axis represents age of brain tissue. Grey bars represent slice cultures subjected to NMDA (300 μM) for 24 h. *$p < 0.05$ compared to 8-9 days old brain tissue, † $p < 0.05$ compared to age matched slice culture, # $p < 0.05$ compared to untreated slice culture.

The NAA/creatine ratio, a frequently used parameter for indication of neuronal loss or dysfunction in MRS, was decreased by 81% in cultured slices treated with

NMDA compared to untreated slices. However, the NAA/creatine ratio was also lowered in untreated cultured slices by 38, 53 and 56% when compared to the NAA/creatine ratio of age-matched hippocampus developed *in vivo* for 15-16, 19-22 and 28-30 days respectively.

Efflux of NAA from hippocampal slices (Paper II and III)

A major new finding in this work was that NAA can be released from neurons by activation of the NMDA receptor. The efflux was prominent from both cultured and acutely prepared hippocampal slices and the major part of the efflux occurred after the NMDA-stimulation (Paper II, Fig. 2 and Paper III, Fig. 3). The efflux was Ca^{2+} dependent and was not affected by hyperosmotic solution (Paper II, Fig. 4 and Paper III, Fig. 3).

When cultured hippocampal slices were subjected to depolarisation by high K^+ or decreased extracellular osmolarity no efflux of NAA was observed while efflux of the osmoregulator taurine was induced by decreased extracellular osmolarity (Paper II, Fig. 4 and Paper III, Fig. 1). NAA tissue-levels were not changed by extended culturing or incubation of slices in moderately decreased extracellular osmolarity while taurine and creatine levels were decreased (Paper III, Fig. 1 and Fig 2.). However, when acutely prepared slices were subjected to a brief and large decrease in extracellular osmolarity an efflux of NAA could be detected. This efflux of NAA was accompanied by efflux of taurine and the putative osmoregulator creatine as well as by efflux of glutamate and GABA (Paper III, Fig. 2 and Table I in this section). The NAA efflux was unchanged by removing extracellular Ca^{2+} and by blocking the NMDA receptor with MK-801 (Paper III, Fig. 2). The temporal pattern of NAA efflux differed from that of taurine and creatine in that taurine and creatine persisted after the efflux (Paper III, Fig. 2 and Table I in this section).

Table I. Individual contribution (%) to the total release of selected amino acids and NAA from hippocampal slices during and after hypoosmotic shock. ND; no data, NE; no efflux above baseline.

	Cultured slices		Acutely prepared slices	
	During	After	During	After
Taurine	64.2	60.5	30.4	47.8
HypoTau	31.0	32.1	NE	NE
Creatine	ND	ND	37.5	50.5
NAA	NE	NE	22.4	NE
PEA	4.3	4.9	2.3	0.3
Aspartate	0.0	0.4	1.0	0.8
Glutamate	0.1	1.4	5.0	NE
GABA	0.5	0.8	1.5	0.6

Depolarisation with high K^+ also resulted in efflux of NAA from acutely prepared slices that was most pronounced after depolarisation. This efflux could be blocked both by MK-801 and by hyperosmotic solution (Paper III, Fig. 3).

Delayed excitotoxicity (Paper II)

Application of NMDA (above 30 μM) for 5 min to cultured hippocampal slices resulted in delayed excitotoxicity. The cell damage increased gradually and after 8 hours the PI intensity in the CA1 area reached significant values (Paper II, Fig. 5) Incubation with kainate (300 μM) for 5 min induced a similar toxicity as NMDA (60 μM) but high K^+ (50 mM) was not toxic when PI uptake was analyzed 24 hours after the challenge (Paper II, Fig. 5). There was a linear correlation between the NMDA-induced efflux of NAA and PI intensity 24 hours later ($R^2 = 0.94$ for CA1 and $R^2 = 0.91$ for CA3) and 10, 20 and 30 μM NMDA were sub toxic concentrations (Fig. 7). However, adding NAA to the culture medium at 10, 5 or 1 mM did not change the PI intensity after 24 hours compared to control slices (Paper II, Fig. 6).

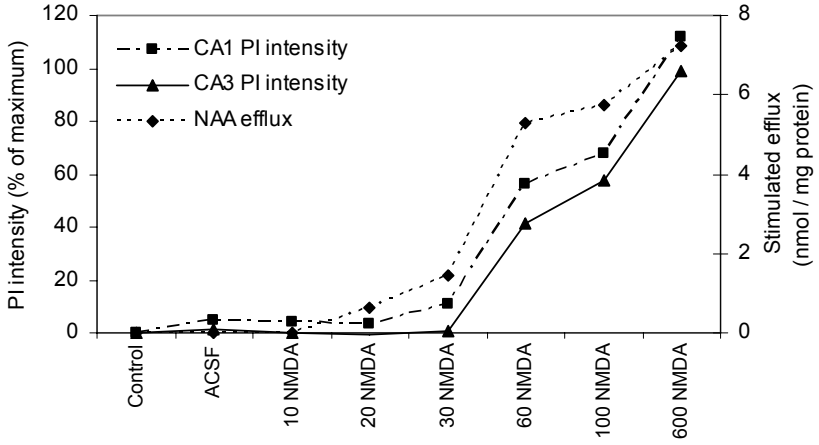

Figure 7. Correlation between efflux of NAA and delayed cell death. NMDA concentrations are in μM.

Effects of increased intracellular NAA (Paper IV)

To be able to study changes in the intracellular concentration of NAA we evaluated the monomethyl ester of NAA (NAA MME) as a way of increasing the low NAA levels in the hippocampal slice cultures. Culturing of slices for 3 days with 30 mM of NAA MME resulted in a six-fold increase in intracellular NAA levels. Culturing with NAA alone did not increase the intracellular levels of NAA significantly (Paper IV, Table I). The procedure was non-toxic as estimated by the lack of PI uptake in NAA MME-treated slices (Paper IV, Fig. 2).

In spite of the major increase in NAA, PEA was the only amino acid studied that showed a compensatory decrease. However, the decrease did not compare with the increase in NAA (Paper IV, Table I). We therefore measured the inorganic anion Cl^- which showed a decrease in NAA MME-treated slices which was on the borderline of significance ($p = 0.06$) (Paper IV, Fig. 3). However, as for PEA the decrease in Cl^- concentration did not match the elevation in NAA concentration.

The position of the carboxyl groups of NAA and earlier studies suggests a potential role as a Ca^{2+} chelator (Rubin et al., 1995a). NAA MME treatment increased NAA in neurons which potentially would make them less vulnerable to excitotoxicity initiated by elevated intracellular Ca^{2+} (Paper IV, Fig. 1). To address this issue NMDA was applied to NAA MME treated slices for 5 min as described above (see page 20 and 21). However, there was no change in delayed excitotoxic cell death in NAA MME treated slices compared to control (Paper IV, Fig. 2).

Effects of NAA on ASPA activity and sulfatide content

Slices cultured for 5 days were added NAA at 1 or 10 mM and cultured for another 13 days. This treatment did not result in any changed ASPA activity or altered production of the myelin lipid sulfatide (Fig. 8). There was however a developmental increase in ASPA as well as sulfatide in the cultured slices (Fig. 8).

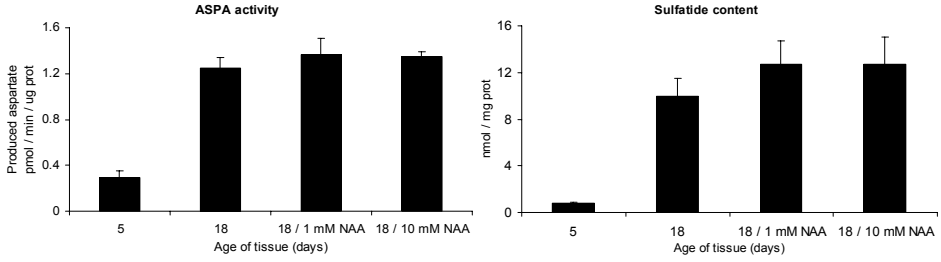

Figure 8. ASPA-activity and sulfatide content in hippocampal slice cultures cultured with NAA for 13 days.

DISCUSSION

HPLC determination of NAA (Paper I)

The use of HPLC for determination of NAA was first described by Koller and co-workers in 1984 (Koller et al., 1984). This is still the most common HPLC method for NAA determination. However, reports of problems with the separation of NAA in medium containing high K^+ and even an unsuccessful attempt to use this method has been described (Taylor et al., 1994; Faull et al., 1999). In view of this, a reversed phase method with UV detection was developed for the present studies. The current method is based on the principle that NAA is neutral at low pH and therefore can be retained on a reversed phase column in aqueous elution buffer.

It is generally not recommended to use pure aqueous buffer in combination with silica based columns. The use of low pH in the buffer could also increase the rate of silica hydrolysis. However, the current column was found to be robust in terms of separation and column life time even without the use of sample purification or extensive washing between injections as has been reported in other HPLC methods with UV detection for the determination of NAA (Koller et al., 1984; Tavazzi et al., 2000).

With regard to NAA analysis by HPLC the current method is comparable or better in sensitivity than previous methods with UV detection (Koller et al., 1984; Tavazzi et al., 2000). The use of HPLC with 2-aminoanthracene derivatization and fluorescence detection increases the sensitivity for NAA determination 10-fold compared to the current method. However, a complicated setup is required and the derivatization step is sensitive to salt-containing solutions such as ACSF (Korf et al., 1991).

It can be noted that reversed phase HPLC has been utilised earlier for NAA determination (Baslow and Yamada, 1997; Nudmamud et al., 2003). However, potential interferences of other organic anions with NAA have to our knowledge not been reported. The present method was evaluated and validated for creatine and a number of other metabolites (including the neuropeptide NAAG) were also separated. The current method can thus be used in studies involving other organic anions than NAA.

Taken together the HPLC method developed for NAA and creatine determination is robust, easy to use, free from co-eluting peaks and comparable and better in sensitivity than previous methods using UV detection.

Content of NAA and amino acids in cultured hippocampal slices and *in vivo* (Paper I)

The established developmental increase in NAA concentration *in vivo* could not be demonstrated in the cultured slices (Fig. 6). This finding is in agreement with an earlier study showing low NAA levels in cultured slices of mouse hippocampus (Baslow et al., 2003). One possible contributing factor to the low NAA is that the high glucose concentration in the culture medium could favour anaerobic metabolism which also is indicated by the immature forms of lactate dehydrogenase as mentioned on page 19 (Schousboe et al., 1993). This could then reduce the synthesis of NAA which is highly dependent on oxidative phosphorylation (Clark, 1998). The lack of excitatory afferents to the cultured slice may also reduce NAA as shown earlier in the visual terminal area after stretch injury to the optic nerve (Rango et al., 1995). An alternative explanation to the low NAA levels could be related to the presence of a glia sheet between the body of the slice and the membrane of the insert (Schultz-Suchting and Wolburg, 1994). The content of NAA in glia is low and expression of NAA concentration in nmol/mg protein would then result in apparently lower values due to a higher contribution of glial proteins to total protein content in the cultured slices compared to *in vivo*. However, the 50-60% lower concentration of NAA in the slices is probably not only due to an increase in glia/neuron ratio as GABA, a predominantly neuronal amino acid, was not reduced after the first week of culturing and reduced to a lower degree than NAA after 2 and 3 weeks of culturing (Fig. 6).

In addition to the change in NAA the concentration of the neurotransmitters glutamate and GABA (after two weeks of culturing) were decreased (Fig. 6). In contrast to GABA, glutamate was not released by depolarisation by high K^+ (Paper II, Fig. 4). This suggests that the glutamate transmission machinery and/or related enzymes are downregulated in the cultured slices. This is in line with the reported decreased expression of AMPA receptors in the hippocampal slice (Fabian-Fine et al., 2000). Alternatively the glutamate uptake is so efficient that no glutamate is allowed to escape to the incubation medium. The use of glutamate-uptake blockers in combination with depolarisation with high K^+ would clarify if glutamate is indeed released by depolarisation in the cultured slices. The hypotaurine concentration was increased dramatically in the cultured slices compared to *in vivo* tissue reaching levels comparable to those of taurine (Fig. 6). The reasons for this were not investigated further but may be related to lower expression of the taurine synthesizing enzyme or alternatively hypotaurine is not converted to taurine via free radicals to the same extent during culturing as *in vivo* (Aruoma et al., 1988). This could in turn be due to lower aerobic energy metabolism as discussed above (see page 19).

The neurochemical differences between the cultured slices and hippocampus developed *in vivo* were not due to ongoing cell death in the cultured slices as slices cultured for up to three weeks showed no extensive PI uptake (data not shown) (Sakaguchi et al., 1997).

The large decrease in NAA concentration (87%) after NMDA-treatment (Fig. 6) is in line with an earlier study showing reduced NAA levels in acutely prepared slices from guinea pig after NMDA treatment (Thatcher et al., 2002). Also, the large decrease in NAA, GABA and glutamate levels after NMDA treatment confirms the expected neuronal localisation of NAA in cultured slices (Fig. 6).

NAA and creatine show strong signals in MRS *in vivo* and decreased NAA/creatine ratios are often interpreted as a reduction in NAA that is secondary to neuronal death or dysfunction. The drawback of the assumption of a stable creatine has been discussed earlier (Ferguson et al., 2002). The present data show that after NMDA-mediated toxicity the decreased NAA/creatine is a good reflection of neuronal cell death (Fig. 6). However, NAA/creatine ratios in viable cultured slices were of the magnitude 50% lower than in the hippocampus developed *in vivo*. This confirms the view that lowered NAA/creatine does not necessarily reflect either neuronal death or dysfunction but may be related to altered energy metabolism (Rango et al., 1995; Clark, 1998; Baslow et al., 2003).

Taken together, there are differences but also similarities in the neurochemistry of the hippocampus developed *in vitro* compared to *in vivo*, suggestive of a partly changed neuronal development. However, the possibility to study extracellular neurochemistry in parallel with long term toxicity make the cultured slices a good complement to acutely prepared slices and *in vivo* studies.

Efflux of NAA from hippocampal slices (Paper II and III)

The major aim of this thesis was to study how the efflux of NAA from neurons is regulated. One obvious reason for this is that many of the proposed functions for NAA require a regulated efflux of NAA (Chakraborty et al., 2001; Baslow, 2002; Gehl et al., 2004). Although efflux of NAA to the extracellular space has been reported *in vivo* the mechanisms have not been determined and *in vitro* efflux of NAA has never been described. (Taylor et al., 1994; Lin et al., 1995; Gotoh et al., 1997; Sager et al., 1999b; Al-Samsam et al., 2000).

Efflux mediated by the NMDA receptor

In the present work it was shown that activation of the NMDA-receptor mediates NAA efflux, both from cultured and acutely prepared hippocampal slices (Paper II, Fig. 2 and Paper III, Fig. 3). In cultured slices efflux also occurred for the anions glutathione and PEA as well as the neutral amino acids taurine and hypotaurine (Paper II, Fig 2.). It is important to realise that the efflux was not due

to unselective membrane rupture. This was clearly demonstrated by the lack of NMDA-mediated efflux of glutamate and GABA which have high intra- to extracellular ratios (Paper II, Fig. 4, Paper III, Fig. 3) (Lerma et al., 1986). Also, comparison of the efflux of amino acids and UV-absorbing species after cell burst (induced by incubation in water) was markedly different compared to efflux mediated by NMDA (Paper II, Fig. 3).

The efflux of NAA is expected to occur mainly from neurons as NAA predominantly is localized to neurons (Fig. 6) and as NMDA receptors to a large extent also are neuronal. It should be taken into account that NMDA receptors have recently been identified on processes of oligodendrocytes (Karadottir et al., 2005; Salter and Fern, 2005). Since oligodendrocytes can contain NAA, efflux from these cells is theoretically possible.

What kind of channels, pores and/or transporters are involved in the efflux?
Volume-regulated anion channels do not appear to be involved in the NMDA-receptor mediated NAA efflux as hyperosmotic medium was ineffective in blocking efflux both from cultured and acutely prepared slices (Paper II, Fig. 4, Paper III, Fig. 3). A previous study from our lab showed a NMDA-mediated efflux of the anions glutathione and PEA which was partly inhibited by the Cl^- channel blocker DNDS and the calmodulin antagonist W7 (Wallin et al., 2003). In the present study glutathione and PEA efflux was also stimulated by NMDA (Paper II, Fig. 2). This is suggestive of a Ca^{2+}-dependent Cl^--channel (CaCC) as a mediator of the efflux. Indeed CaCCs are present in neurons and can be activated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Frings et al., 2000; Nilius and Droogmans, 2003). A CaCC has been shown to be opened by overactivation of glutamate receptors in cultured cortical cells in a delayed manner similar to the delayed maximal efflux of NAA after NMDA-receptor activation observed in our study (Backus and Trube, 1993). The activation of the CaCC was inhibited by NMDA-receptor antagonists and the channel was permeable to organic anions such as acetate and methanesulphonate (Backus and Trube, 1993). Unfortunately no specific blockers for CaCC exist and some Cl^--channel blockers also block the NMDA-receptor (Lerma and Martin del Rio, 1992; Hartzell et al., 2005). It might, however, be possible to use these blockers in a protocol where the blocker is applied after the NMDA-receptor activation.

Possible functions of NMDA-mediated efflux of NAA
As mentioned above many of the proposed functions for NAA require a regulated NAA efflux. The involvement of the NMDA receptor opens up for some interesting speculation. The use of NAA in myelin lipid synthesis has been shown in several studies as mentioned on page 14. High neuronal activity leads to activation of the NMDA receptor which potentially could release NAA to the extracellular space also *in vivo*. Recently, NAA and ASPA in oligodendrocytes were

shown to be important for regulation of the expression of the neurotropic factor BDNF in neurons. BDNF in turn regulates oligodendrocyte differentiation (Francis et al., 2006). In combination with the recent finding that ASPA is present in the nucleus of oligodendrocytes (Hershfield et al., 2006) it is tempting to speculate that NAA released from neurons by activation of the NMDA-receptor during development can be a signal to oligodendrocytes to adapt to neuronal activity by producing myelin and/or to differentiate. In our preliminary data no change in ASPA activity or sulfatide content was found when NAA was applied to cultured slices for 13 days (Fig. 8). This suggests that excessive extracellular NAA alone does not affect differentiation or proliferation of oligodendrocytes.

NAA released by high neuronal activity could also be taken up by astrocytes and be used as a substrate for NAAG synthesis (Sager et al., 1999a; Gehl et al., 2004; Fujita et al., 2005). NAAG could in turn communicate back to neurons by its feature as an NMDA-antagonist and agonist of metabotropic receptors as has been proposed earlier (Baslow, 2000).

NAA is increased in Canavan disease which primarily affects white matter regions in the brain (Matalon et al., 1995). In our studies release of NAA correlated with delayed cell death (Paper II and Fig.). However, application of NAA to the culture medium for 13 days was non-toxic and did not change the slice content of ASPA or sulfatide which are both associated with oligodendrocytes (Fig. 8). Although the time scale *in vitro* is very different from development of Canavan disease in humans the results indicates that the high concentration of NAA *per se* does not contribute to the pathology in Canavan disease.

Efflux mediated by changes in extracellular osmolarity

In the present work no release or tissue decrease in NAA was observed in hippocampal slice cultures subjected to decrease in external osmolarity (Paper III, Fig. 1). However, efflux and tissue decrease of the established osmoregulator taurine and the putative osmoregulator creatine was induced by decreased extracellular osmolarity (Paper III, Fig. 1) (Bothwell et al., 2001; Ordaz et al., 2004). It can be noted that in addition to taurine and creatine a large amount of hypotaurine was released and lost from the cultured tissue by decreased extracellular osmolarity (Paper III, Fig. 1). The involvement of hypotaurine in volume regulation has been suggested from studies in brain slices of rat cortex (Bothwell et al., 2001). Hypotaurine is also a well established osmolyte in certain marine invertebrates (Yin et al., 2000).

In contrast to cultured slices a brief acute hypoosmotic shock resulted in efflux of NAA together with glutamate, GABA and PEA from acutely prepared slices (Paper II, Fig 2 and Table I on page 27). The results compare well with those of Bothwell and co-workers who reported on large changes in intracellular levels of a

number of amino acids, including NAA, following a 30 min incubation of cortical slices in hypoosmotic medium (100 mOsm reduction) (Bothwell et al., 2001). The temporal pattern of the efflux of the anions NAA, glutamate and PEA differed from that of the neutral amino acids taurine and creatine which persisted after the hypoosmotic shock (Paper III, Fig. 2 and Table I on page 27). Efflux of NAA, glutamate and PEA may therefore be used as a complement to taurine and creatine to reduce an acute decrease in extracellular osmolarity. Efflux of taurine and creatine as well as hypotaurine, if it is present, may then be involved in volume decrease after the hypoosmotic challenge. This reasoning is in line with earlier results in hippocampal slices which showed rapid efflux of transmitter amino acids but a prolonged efflux of taurine following hypoosmotic stress (Franco et al., 2001). Also, efflux of $^{125}I^-$ in neuronal cell lines in response to a hypoosmotic shock was rapid and transient whereas that of taurine was more prolonged (Moran et al., 1997). It can be noted that neither the efflux channel nor the sensing factor responsible for release of taurine has been determined (Stutzin and Hoffmann, 2006). Likewise the channel that mediates efflux of NAA during the hypoosmotic shock is unknown.

The fact that NAA was released by hypoosmotic shock suggests a potential role in osmoregulation. However, the brain will never sense such a dramatic decrease in osmolarity as -166 mOsm (Andrew et al., 1997). When a more physiological and extended decrease in extracellular osmolarity was used no NAA was lost from the tissue in contrast to taurine which was decreased (Paper III, Fig. 1 and 2). In addition, NAA has bee reported to have a relatively small contribution to osmoregulation during hyponatremia compared to K^+, Cl^- and amino acids (Pasantes-Morales et al., 2000). In view of the efflux of NAA by the NMDA receptor it is interesting to note that hypoosmolarity and hyponatremia also decreased tissue glutamate which indicates that glutamate is released by these treatments (Sterns et al., 1993; Bothwell et al., 2001). Released glutamate by hypoosmotic conditions could thus hypothetically stimulate efflux of NAA via activation of the NMDA receptor. However in our case this does not appear to be the case as the NMDA-receptor blocker MK-801 did not affect efflux caused by hypoosmotic shock (Paper III, Fig. 2). Efflux of NAA was stimulated by hypoosmotic shock from acutely prepared slices but not from cultured slices (Paper III, Fig. 1 and 2). One reason could be that the sum of the concentrations of neutral amino acid osmolytes in cultured slices, i.e. hypotaurine, taurine and creatine is much higher than the corresponding value of taurine and creatine in acutely prepared slices. In cultured slices it may thus not be "necessary" to release NAA because the efflux of the neutral amino acids is so high. Another reason may be that the NAA concentration is much lower in cultured slices and as proposed earlier is downregulated as a neuronal osmolyte (Baslow et al., 2003).

Efflux mediated by depolarisation

In the present work no role for NAA as an osmoregulator during moderate decreases in extracellular osmolarity was found. In contrast to cultured slices NAA was released after depolarisation by high K^+ from acutely prepared slices as has been described earlier *in vivo* (Taylor et al., 1994; Lin et al., 1995; Davies et al., 1998). The depolarisation-mediated efflux of NAA by high K^+ was blocked both by the NMDA-receptor antagonist MK-801 and hyperosmotic incubation medium suggesting two modes of NAA release; one swelling dependent and one NMDA-receptor dependent (Paper III, Fig. 3). An intriguing finding is that hyperosmotic solution also decreased the efflux of glutamate after depolarisation of acutely prepared slices while GABA efflux was unchanged (Paper III, Fig. 3). The reason for this decrease is unknown but it can be speculated that by high K^+ induces swelling of astrocytes followed by efflux of glutamate which can be inhibited by hyperosmotic solution. Consequently NAA efflux in response to high K^+ may in part be due to NMDA-receptor activation by glutamate released from astrocytes (Bak et al., 2004; Kimelberg, 2005).

Effects of increased intracellular NAA

N-acetylaspartate monomethyl ester

NAA concentration in cultured hippocampal slices was increased by treatment with NAA MME (Paper IV, Table I). Breakdown of NAA MME liberates NAA and methanol but PI uptake was unchanged in slices treated with NAA MME compared to control slices, showing that the intracellular increases in methanol and NAA were non-toxic. In the current work we did not evaluate the culturing time needed to obtain increased NAA levels. However loading of acutely prepared slices with NAA MME showed a 2-fold increase of intracellular NAA concentration after 2 hours of incubation (data not shown). The use of the dimethyl ester would probably enhance the uptake but would in turn generate more methanol per NAA liberated. The solubility of the dimethyl ester in water is probably also much lower due to its lack of charge. The NAA increase observed in slices after NAA MME treatment most likely originates from hydrolysis of NAA MME inside the cells. There is a possibility that some of the ester is left unhydrolysed in the cell and then converted to NAA by the extraction procedure which involves homogenisation by acidic solution (0.3 M $HClO_4$). However, in the initial studies 90% methanol, a milder extraction medium, was used for extraction and this generated a similar increase in NAA after NAA MME treatment as that obtained with the $HClO_4$ extraction procedure. This demonstrates that the increase in NAA concentration most likely is due to intracellular hydrolysis of NAA MME.

The NAA MME does not discriminate between the different types of cells in the slice. The increase of NAA in neurons is suggested by the higher efflux of NAA

from NAA MME treated slices compared to untreated slices after NMDA receptor stimulation (Paper IV, Fig. 1).

Effect of increased intracellular NAA on anions

The increase in NAA after NAA MME treatment was about 6 times that of control slices (Paper IV, Table I). The concentration increased from about 3 mM to 18 mM (assuming a protein/wet weight ratio of 10%). Of all the amino acids studied only a decrease in PEA of about 0.3 mM was demonstrated. The decrease in Cl^- concentration was about 2.5 mM. This leaves about 24 mM (12 mM NAA) of negative charge that has to be compensated for. One regulatory mechanism may be an increase in intracellular K^+ by 24 mM which would result in a total increase in intracellular osmolarity by 36 mOsm. We did not measure the concentration of K^+ or anions such as HCO_3^- or PO_4^{3-} and a compensatory decrease in any of these can not be occluded. An increased intracellular osmolarity would result in cell swelling but if so, it appears non-toxic as indicated by the lack of PI uptake (Paper IV, Fig. 2).

The tendency towards a decrease in Cl^- is interesting in view of the function of NAA as an important contributor to anion homeostasis. It is interesting to note that the developmental increase in NAA and decrease in intracellular Cl^- concentration parallel each other. The decrease in Cl^- concentration changes the effect of GABA from excitatory to inhibitory and is dependent on the expression of Cl^- pumps such as KCC2 (Ben-Ari, 2002). The reverse, i.e. that GABA changes from inhibitory to excitatory, has been shown to occur after various trauma (van den Pol et al., 1996; Khalilov et al., 2003). Interestingly trauma also lowers NAA levels, again pointing towards the intriguing apparent correlation between high NAA and low intracellular neuronal Cl^- (Tsai and Coyle, 1995). If changes in NAA concentration affect the Cl^- concentration, and thus the effect of GABA, requires further studies using electrophysiological techniques such as perforated patch clamp in combination with varying intracellular NAA via the loading technique with NAA MME.

NAA as a Ca^{2+} chelator

The influx of Ca^{2+} through the NMDA receptor is well established as an initiator of excitotoxic cell death (Fig. 1) (Sattler and Tymianski, 2000). The unchanged excitotoxic cell death in slices treated with NAA MME compared to control slices does not speak in favour for NAA as an important intracellular Ca^{2+} chelator. Although NAA is also released by NMDA-receptor activation which thus would reduce its putative chelating property, the delayed form of the efflux predominates (Paper II, Fig. 2 and Paper III, Fig. 3) which assures an increased intracellular NAA level in NAA MME treated slices during NMDA-mediated Ca^{2+} influx.

CONCLUSIONS

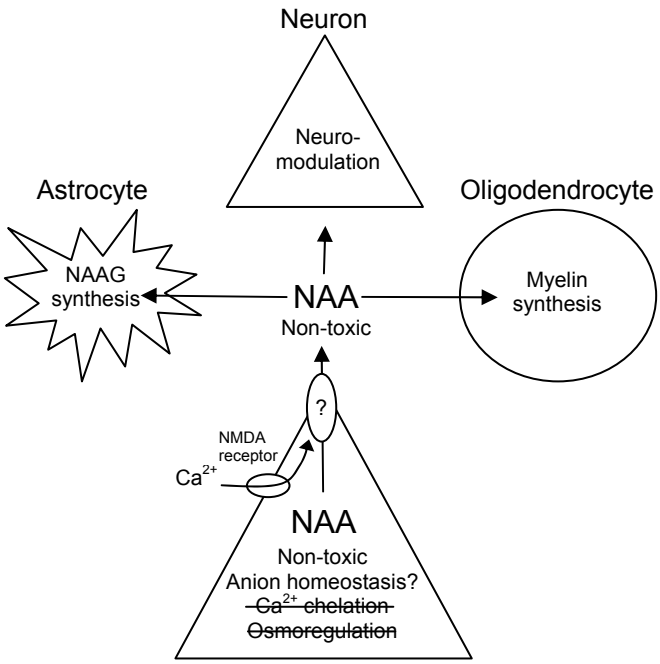

Figure 9. Schematic picture of the conclusions drawn from the results in this thesis. NAA efflux from neurons is regulated by the NMDA receptor. Increased intra- and extracellular NAA is non-toxic and NAA is not a Ca^{2+} chelator or a major osmoregulator in neurons. NAA may be involved in neuronal anion homeostasis.

The most important finding in this work is that NAA can be released from neurons by activation of the NMDA receptor (Fig. 9). The efflux is dependent on extracellular Ca^{2+} and occurs both by toxic and non-toxic receptor-activation. The fact that the NMDA receptor is involved in the efflux of NAA is interesting in relation to a number of the proposed functions of NAA. For example NAA released by neuronal activity can be used by oligodendrocytes for myelin production or used by astrocytes for NAAG synthesis (Fig. 9). Future studies needs to clarify if NAA released from neurons by the NMDA receptor is incorporated into myelin or NAAG. It is also obvious that the channels involved in transport of NAA out of neurons needs to be identified.

It was also shown in this work that high extra- and intracellular NAA concentrations are non-toxic (Fig. 9). This can have important implications for elucidating the mechanisms underlying Canavan disease. From this work it is

suggested that the increased NAA levels observed in Canavan disease is not responsible for the pathology.

The function of NAA as an important Ca^{2+} chelator is also questioned by the current results which showed no protective effects of increased NAA levels on development of delayed Ca^{2+} dependent excitotoxicity (Fig. 9). Also the function of NAA as an osmoregulator when extracellular osmolarity is reduced could not be concluded by the results in this thesis (Fig. 9). NAA can obviously be released by large decreases in extracellular osmolarity but this is a situation that the normal brain will not sense, even under pathological situations.

The increase in NAA levels by NAA MME can be a useful tool in future *in vitro* studies on NAA metabolism and function.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärnan är vårt mest komplicerade organ men ännu är det mycket som man inte vet om dess funktion. En molekyl som förbryllat hjärnforskarna i nästan 50 år är N-acetylaspartat (NAA). Trots att NAA finns i lika hög koncentration som en liknande och mycket viktig molekyl, signalsubstansen glutamat, har man inte lyckats bestämma funktionen av NAA. Det finns dock många intressanta företeelser med NAA som tyder på att den har en viktig funktion i hjärnan. NAA har t.ex. en strikt uppdelad metabolism; den tillverkas exklusivt i neuroner, de celler som leder nervimpulser, och bryts ner endast i hjärnans stödjeceller, gliacellerna. Varför denna uppdelning finns vet man inte men den kräver att NAA frisätts från neuronen på ett kontrollerat sätt.

I min avhandling har jag visat att NAA kan frisättas via aktivering av NMDA-receptorn. NMDA-receptorn är en viktig receptor som aktiveras vid nervtransmission men som också kan ge upphov till cellskada om den överaktiveras, s.k. excitotoxicitet.

Vid överaktivering av NMDA-receptorn och stor frisättningen av NAA skulle man kunna tänkas sig att NAA bidrar till excitotoxiciteten. Det verkar dock inte som detta är fallet eftersom tillsatts av NAA till hjärnvävnad inte är skadande. En funktion av frisättning av NAA vid nervtransmission kan vara att fungera som en signal till gliaceller. Gliacellerna kan då förändra sig så att de t.ex. underlättar nervtransmissionen. En sådan funktion av NAA stöds av det faktum att den kan användas av gliaceller för uppbyggnad av myelin, ett ämne som fungerar som isolering av nervtrådar. Dessutom verkar nedbrytningen av NAA av gliaceller vara nödvändig för överlevnad eftersom barn med Canavans sjukdom, som just beror på att nedbrytningen inte fungerar, avlider innan de fyllt 10 år. Frisättning av NAA verkar alltså vara viktigt för normal hjärnfunktion

Dessutom har jag visat att NAA kan frisättas vid extrema svullnad av nervceller. Frisättningen av NAA vid denna situation sker förmodligen för att få cellerna att krympa tillbaka till sin normala storlek. Detta sker eftersom NAA tar med sig lite av det vatten som fått cellen att svälla när den frisätts. Extrem svullnad av celler sker dock aldrig i hjärnan och vid normal svullnad av nervceller såg jag ingen frisättning av NAA. Mina resultat ifrågasätter därför den föreslagna rollen av NAA som en viktig komponent vid normal volymreglering av celler i hjärnan.

En av anledningarna till att funktionen av NAA inte fastställts är att det är svårt att på konstgjord väg manipulera koncentrationen av NAA. Man har alltså inte direkt kunnat studera vad förändrade NAA nivåer innebär och på så sätt fått reda på dess funktion kan vara. För att lösa detta problem har använde jag mig av ett ämne som strukturellt liknar NAA. Detta ämne kunde omvandlas till NAA inuti celler och på

så sätt blev det möjligt att öka NAA nivåerna i vävnad. Ökningen av NAA var inte toxisk i sig och när hjärnvävnad med ökat NAA innehåll utsattes för excitotoxicitet, som är en vanligt förekommande form av toxicitet i t.ex. stroke, var celldöden oförändrad. Detta visar att en ökning NAA i neuron inte heller är toxiskt men å andra sidan inte heller skyddar mot toxicitet.

Sammantaget har jag visat att NAA kan frisättas på två sätt från neuron och att höga nivåer av NAA i och utanför cellen inte är toxiskt. Detta är viktig information som kan användas i framtida studier av funktionen av NAA och som i sin tur kan leda till läkemedel mot den dödliga Canavans sjukdom.

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APPENDIX (PAPER I-IV)