AMINO ACID NEUROTRANSMISSION AND ITS REGULATION BY VALPROATE: FOCUS ON ASPARTATE

PhD thesis

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2011

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1275

ISBN 978-82-8264-299-6

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

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ACKNOWLEDGMENTS

Most of the work presented in this thesis was carried out at the Department of Anatomy, Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo and the Center for Molecular Biology and Neuroscience (CMBN), although some of the studies started at the Norwegian Defense Research Establishment (FFI). I am grateful to the University of Oslo for providing research facilities and financial support, making this PhD project possible, and to FFI for allowing me to use their laboratory facilities whenever needed.

I would like to thank my supervisor Dr. Vidar Gundersen for sharing his expertise with me and for giving me the academic freedom set up my own experiments, yet bringing me back to earth when my hypothesis were to far fetched. You have given me invaluable guidance and advice, and without you this thesis would not have been possible.

I would also like to thank the previous head of our laboratory, Prof. Emeritus Jon Storm-Mathisen for welcoming me to the Synaptic Neurochemistry Laboratory, for sharing his knowledge with me and for always looking at things from the positive side. I would like to thank the current head of our laboratory, Associate Prof. Linda Hildegard Bergersen, for all the effort she has put into creating an encouraging and enthusiastic working environment.

I am grateful to my co-supervisor, Dr. Bjørnar Hassel, who introduced me to the exciting field of neuroscience when I first started as a master student in his lab. Thank you for fruitful discussions and for sharing your excellent scientific knowledge, experience and enthusiasm!

To all my current and previous colleagues at the Department of Anatomy who are contributing to a nice and inspiring environment: It has been a pleasure working with you! A special thank goes to Kaja Nordengen for excellent collaboration during the last year of my thesis, and to Maja Puchades for comments on the manuscript.

I would like to thank all the co-authors: Max Larsson, Aleksander Talgøy Holten, Evy Grini Iversen, Kaja Nordengen, Karen A. Boldingh Debernard, Laura M. Prolo and Richard Reimer, who have contributed to this work in different ways.

A warm thank you goes to my family and friends, who have supported me throughout the process of this PhD work. Especially, I would like to thank Bjørn, Christoffer and Thomas for being patient and understanding when I have spent long hours in the lab, and for reminding me that there are more important things in life than counting gold particles…

Cecilie Morland

Oslo, December 2011

CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following original papers:

Paper I Holten AT*, **Morland C***, Nordengen K, and Gundersen V. (2008) Vesicular release of L- and D-aspartate from hippocampal nerve terminals: immunogold evidence. The Open Neurosci. J. 2:51-58.
* *Holten and Morland contributed equally to the work*.

Paper II **Morland C,** Nordengen K, Larsson MD, Prolo LM, Reimer R, Gundersen V. (2011) Vesicular uptake and exocytosis of aspartate is independent of sialin. Submitted.

Paper III **Morland C**, Nordengen K, Gundersen V. (2011) Valproate causes reduction of the excitatory amino acid aspartate in nerve terminals. Under review, second round; Neuroscience Letters.

Paper IV **Morland C**, Boldingh KA, Iversen EG, Hassel B. (2004) Valproate is neuroprotective against malonate toxicity in rat striatum: an association with augmentation of high-affinity glutamate uptake. J Cereb Blood Flow Metab. 24:1226-34.

Additional papers during the PhD period:

Bergersen LH, **Morland C**, Ormel L, Rinholm JE, Larsson M, Wold JFH, Røe ÅT, Stranna A, Santello M, Bouvier D, Ottersen OP, Volterra A, Gundersen V. (2011)
Immunogold detection of L-glutamate and D-serine in small synaptic like microvesicles in adult hippocampal astrocytes. Cerebral Cortex.

Larsson M, Sawada K, **Morland C**, Hiasa M, Ormel L, Moriyama Y and Gundersen V. (2011) Functional and anatomical identification of a vesicular transporter mediating neuronal ATP release. Cerebral Cortex.

Larsson M, **Morland C**, Poblete-Naredo I, Biber J, Danbolt NC and Gundersen V. (2011) The sodium dependent inorganic phosphate transporter SLC34A1 (NaPi-IIa) is not localized in the mouse brain: a case of tissue-specific antigenic cross-reactivity. J. Histochem. Cytochem. PMID: 21606201

Nguyen NH, **Morland C**, Gonzalez SV, Rise F, Storm-Mathisen J, Gundersen V, Hassel B. (2007) Propionate increases neuronal histone acetylation, but is metabolized oxidatively by glia. Relevance for propionic acidemia. J Neurochem. 101:806-14

Morland C, Henjum S, Iversen EG, Skrede KK, Hassel B. (2007)
Evidence for a higher glycolytic than oxidative metabolic activity in white matter of rat brain. Neurochem Int. PMID: 17316901

ABBREVIATIONS

AAT	Aspartate aminotranferase
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid
ATP	Adenosine triphosphate
bcl-2	B-cell lymphoma 2
BK_{Ca}	Big potassium channels, calcium activated
BoNT	Botulinum toxin
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CSF	Cerebrospinal fluid
Cx43	Connexin 43
EAAT	Excitatory amino acid transporter
GABA	γ-amino butyric acid
GLAST	Glutamate aspartate transporter
GLT	Glutamate transporter
GS	Glutamine synthetase
HSP-70	Heat shock protein, 70kDa
KB-R7943	2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate
MCT	Monocarboxylate transporter
MK-801	(5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate
NBQX	2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide
NMDA	N-methyl-D-aspartate
NPPB	5-Nitro-2-(3-phenylpropylamino)benzoic acid
PAG	Phosphate activated glutaminase
p-ERK	Phosphorylated extracellular signal-regulated kinase
SDH	Succinate dehydrogenase
SNAP-25	Synaptosomal-associated protein 25
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SRF	Sustained repetitive firing

TBOA	threo-β-Benzyloxyaspartic acid
TCA	Tri-carboxylic acid
TRPC	Transient receptor potential cation channels
TTX	Tetrodotoxin
VEAT	Vesicular excitatory amino acid transporter
VGLUT	Vesicular glutamate transporter
VRAC	Volume-regulated anion channel

INTRODUCTION

The Synapse

By 1850, most biologists recognized cells as the basic unit in living tissue, however there was a debate as to whether the “cell theory” also applied to the brain. The existence of the nerve cells (neurons) had already been described, but the question of whether nerve fibres from different nerve cells were directly connected remained disputed during the late 19th century. Camillo Golgi promoted the “reticular theory”, stating that neurons fused at their junctions and thereby formed a continuous network, a reticulum. The conflicting theory, later known as the “neuron doctrine”, was first expressed by the Norwegian explorer Fridtjof Nansen, who argued that neurons were separated by a gap. He recognized the contact points between nerve cells as the "principal seat of the nervous activity (...) principal seat of intelligence" (Nansen, 1887). However, it was the Spanish neuroanatomist Santiago Ramón y Cayal who, during the debate with Golgi, took a leading role in defending the “neuron doctrine”. Today we understand that Ramón y Cayal and Golgi were actually discussing the existence of synapses.

Although the gap between neurons, the synaptic cleft, could not bee seen until the invention of the electron microscope in the 1950s, the notion “synapse” was introduced by Charles Scott Sherrington in 1906. The same year, Ramón y Cayal and Golgi shared the Nobel Prize in medicine for their work on the anatomy of the nervous system. By that time, Ramón y Cayal ’s “neuron doctrine” was accepted by most scientists, although Golgi clamed in this Nobel lecture that “this doctrine is generally recognized to be going out of favour” (Golgi, 1906).

Nansen and Ramón y Cayal were right abiout the existence of a gap between the neurons. The synapse, consisting of the presynaptic nerve terminal, the postsynaptic element and the gap between them, the synaptic cleft, is now known to be fundamental in neuron-to-neuron signaling. The nature of this signal transmission, however, remained largely unresolved until 1921. Then the German pharmacologist, Otto Loewi, performed his famous experiments confirming that neurons communicate by releasing chemicals, for which he was rewarded the 1936 Nobel Prize in Medicine. Ever since the time of Loewi,

indentifying the signaling molecules used by neurons, understanding normal synaptic function as well as regulation of synaptic activity in response to usage, stimuli and diseases have been a major focus in Neuroscience. The present thesis forms no exception.

Glutamate has been generally accepted as a neurotransmitter since the 1980s, although excitation of neurons by glutamate had been demonstrated electrophysiologically about 20 years earlier (Curtis and Watkins, 1960). The production of antibodies against amino acids (Storm-Mathisen et al., 1983) made it possible to study their location in the brain, and was important in establishing glutamate as a signaling molecule, a neurotransmitter, in the brain. Later, glutamate was demonstrated to be the main excitatory neurotransmitter (for review, see Fonnum, 1984; Ottersen and Storm-Mathisen, 1984b) and the majority of neurons use glutamate as their main transmitter. In addition to glutamate, γ-amino butyric acid (GABA) (Storm-Mathisen et al., 1983) and glycine (Dale et al., 1986) were also established as neurotransmitters. Both of these are inhibitory, GABA predominantly in the brain and glycine in the spinal cord.

Here we investigate whether another amino acid, aspartate, can be used as a neurotransmitter in central nervous synapses (papers I and II). Aspartergic neurotransmission has been debated throughout the last 30-40 years, and will be discussed in more details later.

Synaptic neurotransmission, a highly complex process in the brain, is often altered during disease and can be manipulated with neuropharmaca. Papers III and IV of the present thesis focus on how the neuro-active amino acids, glutamate, aspartate and GABA, can be regulated by an antiepileptic and mood stabilizing drug, valproate.

Cell Types in the Brain

In addition to neurons, the brain contains ependymal cells and epithelial cells of the choroid plexus, cells of the vascular wall (endothelial cells), and glial cells: astrocytes, oligodendrocytes, and microglia. The human brain has been estimated to contain ~10^{11} neurons (Jessell and Kandel, 1993), and these are classified based on which major

neurotransmitter they use. The majority of neurons in the brain are glutamatergic (excitatory), and this group might account for up to 80% of all neurons (Ottersen and Storm-Mathisen, 1984a). The second largest group of neurons consists of inhibitory, GABAergic, neurons. However, both glutamatergic and GABAergic neurons can store and release other transmitters as well, from the same nerve terminals (for review, see El Mestikawy et al., 2011). The astrocyte/neuron-ratio varies between brain regions, but in the human brain, astrocytes outnumber neurons in a 10:1 propotion (Bignami et al., 1991).

Excitotoxicity and Glutamate Receptors

Glutamate is the predominant excitatory neurotransmitter in the brain (Fonnum, 1984; Ottersen and Storm-Mathisen, 1984b), and regulation of glutamate levels is pivotal to maintain normal brain function. The presence of glutamate in the extracellular space at excessive concentration or for extended periods of time can lead to neuronal death. This phenomenon was first discovered in the retina in 1957 (Lucas and Newhouse, 1957), although the name ‘‘excitotoxicity’’ was not introduced until later (Olney, 1969). A classic model for excitotoxicity is excessive stimulation of the glutamate receptors due to elevated extracellular glutamate concentration. The glutamate receptors are primarily localized in the postsynaptic membranes or, at lower densities, in the presynaptic membranes and extrasynaptically on dendrites and astrocytes. The receptors are divided into two major categories; the ionotropic receptors which gate transmembrane ion channels, opening them upon binding of the neurotransmitter, and the metabotropic receptors which, upon transmitter binding, trigger intracellular signalling cascades. The ionotropic glutamate receptors are further divided into three categories according to their ligand selectivity: 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) receptors, kainate receptors, and N-methyl- D-aspartate (NMDA) receptors. All of these are directly linked to cation channels. The AMPA and kainate-receptors gate ion channels that are permeable to potassium and sodium ions, and are responsible for a fast depolarization of the postsynaptic membrane. Some AMPA receptors are also permeable to calcium ions, but during development, the Ca^{2+} permeable subtype of AMPA receptors is replaced by the Ca^{2+} impermeable subtype. Thus, in the adult brain, almost all AMPA receptors are Ca^{2+} impermeable. NMDA receptors, on the other hand, are highly permeable to Ca^{2+}. Due to a

voltage sensitive magnesium block of the NMDA-linked ion channel (Mayer et al., 1984; Nowak et al., 1984), opening of these channels normally requires that the membrane is already depolarized. Thus the NMDA receptors are believed to be responsible for a slower response, one that is dependent on parallel activation of other glutamate receptors. Although the non-NMDA receptors can contribute to excitotoxicity, the main effect seems to be mediated through NMDA receptors (Hahn et al., 1988; Sucher et al., 1991). NMDA receptors play a central role in a number of physiological processes, including long-term potentiation in the hippocampus (Collingridge et al., 1983; Harris et al., 1984; Morris et al., 1986; Wigström et al., 1986; Larson and Lynch, 1988) and synaptogenesis (Kitayama et al., 2003; Manent et al., 2005; Ghiani et al., 2006). However, excessive NMDA receptor activation has been implicated in the pathophysiology of both acute incidents like ischemia (Arundine and Tymianski, 2004), or severe epilepsy (Ghasemi and Schachter, 2011) and in chronic neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and Huntington's disease (for review, see Kalia et al., 2008).

Synaptic Glutamate Release

Synaptic vesicles are membrane enclosed organelles, ~40 nm in diameter, that accumulate and store neurotransmitters. The vesicular uptake of all known neurotransmitters is driven by a vacuolar, ATP consuming, proton pump which generates an electrochemical gradient across the vesicular membrane (Maycox et al., 1988). The vesicular glutamate transporters (VGLUTs), which are responsible for the uptake of glutamate into synaptic vesicles, use the membrane potential established by the proton-ATPase as their driving force. To allow for rapid regeneration of releasable neurotransmitter, the synaptic vesicles undergo a tightly regulated trafficking cycle. Vesicles that are filled with neurotransmitter dock at the active zone, where they undergo priming, making them competent for rapid fusion-pore opening upon the arrival of a calcium signal. Glutamatergic neurotransmission is initiated when an action potential triggers exocytosis of glutamate-containing synaptic vesicles at the active zone of a presynaptic terminal. Exocytosis of synaptic vesicles requires the tightly regulated action of SNARE proteins. Synaptic vesicles can endocytose and be recycled by

three alternative pathways (Reviewed in Sudhof, 2004); (1) Classic endocytosis via clathrin-coated pits adjacent to the active zone (Jarousse and Kelly, 2001; Voglmaier and Edwards, 2007), followed by translocation to the interior of the cell. The vesicles then reacidify and refill with neurotransmitters, either directly or after passing through an early endosomal intermediate. Alternatively (2), after undocking, the vesicles may be reacidified and refilled locally (the "kiss-and-run" model for neurotransmitter release) or finally (3) recently it is proposed that the synaptic vesicles can be refilled without undocking from the plasma membrane (the "kiss-and-stay" model). The fast pathways, (2) and (3), are preferentially used for the rapid recycling of neurotransmitters into the ready releasable pool at low stimulation frequencies, while the slower clathrin-dependent pathways, are active at higher stimulation frequencies, and recruit the recycling- and reserve pools of synaptic vesicles.

Glutamate Uptake and the Glutamate-Glutamine Cycle

An important defense against excitotoxicity is to keep the extracellular levels of glutamate low (at lower μM concentrations). This job is largely done by the excitatory amino acid transporters (EAATs). These are high affinity transporters situated in the plasma membrane of neurons and astrocytes. They can transport glutamate across the cell membranes against a glutamate gradient of several thousand fold (Danbolt, 2001). Five such EAATs have been characterized: EAAT1 (GLAST; Storck et al., 1992; Tanaka, 1993), EAAT2 (GLT Pines et al., 1992), EAAT3 (EAAC Kanai and Hediger, 1992), EAAT4 (Fairman et al., 1995), EAAT5 (Arriza et al., 1997), of which the first three are localized throughout the entire brain, EAAT4 is mainly found in the cerebellum, and EAAT5 is exclusively expressed in the retina.

To ensure efficient glutamatergic signalling, it is essential that glutamate levels are kept low when no signal is transmitted, i.e. to ensure a high signal/noise response of glutamate receptors. Since glutamate is not degraded extracellularly, the glutamatergic signal is terminated when the transmitter is taken up into brain cells via the EAATs. Three different compartments are involved in the removal of glutamate from the synaptic cleft. Uptake into the presynaptic terminal (Gundersen et al., 1993; 1996), probably through EAAT2

(Furness et al., 2008), allows glutamate to be taken up into synaptic vesicles (Naito and Ueda, 1983), and reused as a neurotransmitter. Another possibility is uptake into the postsynaptic dendrite. This is believed to occur through EAAT3 in several brain regions or through EAAT4 in the cerebellum. In this case glutamate is lost from neurotransmission, but can be used in the energy metabolism. This loss of neurotransmitter must be compensated for, otherwise the neurons will be drained of tricarboxylic acid (TCA) cycle intermediates (from which glutamate, GABA and aspartate are formed). Anaplerosis, the formation of TCA cycle intermediates from substances that are not themselves TCA cycle intermediates, may be of importance to maintain energy metabolism and glutamate level during neurotransmission (for review, see Hassel, 2000).The bulk glutamate uptake, however, is into perisynaptic astrocytes via the astrocytic transporters, EAAT1 or -2, and this is the first step of the glutamate-glutamine cycle between neurons and astrocytes (figure 1), of which the purpose is recirculation of neurotransmitter glutamate. Astrocytes, but not neurons express glutamine synthetase (GS), an enzyme able to convert glutamate to glutamine, and found to be essential for production of releasable glutamate in nerve terminals (Laake et al., 1995). From the astrocytes, glutamine is released through system N glutamine transporters to the extracellular space (Chaudhry et al., 1999; 2001; Boulland et al., 2002; 2003), where it is made available for system A glutamine transporters on the neuronal membrane (Jenstad et al., 2009). In the nerve terminal, the mitochondrial enzyme, phosphate-activated glutaminase (PAG) converts glutamine into glutamate (Kvamme et al., 2008), which can be transported into synaptic vesicles by the VGLUTs and thus be ready for synaptic release.

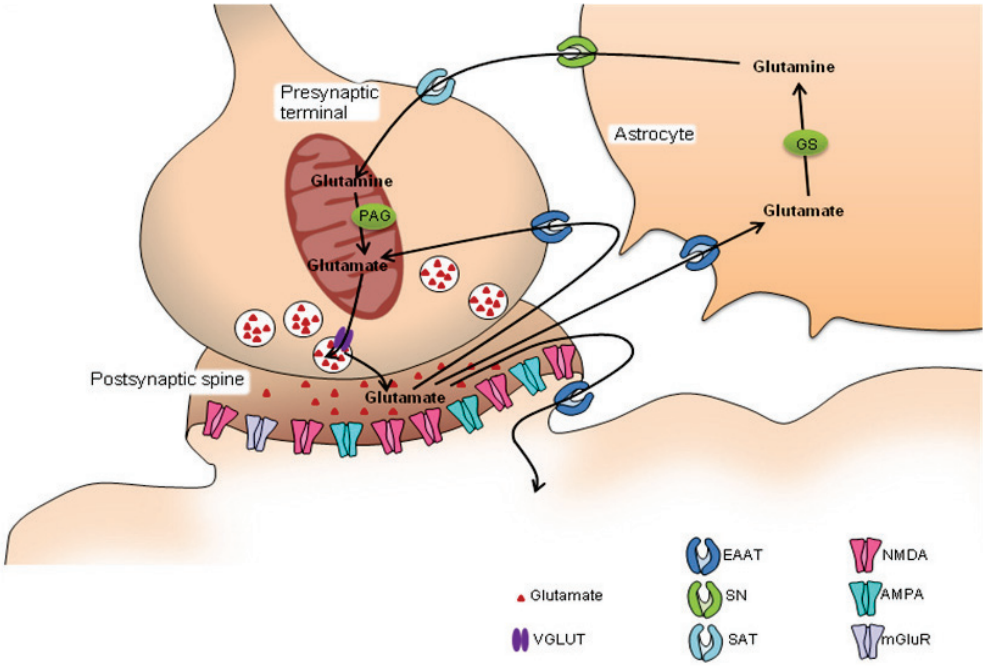

Figure 1: Glutamate–glutamine cycle

VGLUT: vesicular glutamate transporter; EAAT: excitatory amino acid transporter; SN: system N glutamine transporter; SAT: system A glutamine transporter; NMDA: N-methyl-D-aspartate; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor ; mGluR: metabotropic glutamate receptors; PAG: phosphate-activated glutaminase; GS: glutamine synthetase.

The Release Mechanism for Aspartate is Debated

The role of aspartate as a classical neurotransmitter in the brain is controversial, since direct evidence for synaptic vesicle uptake of aspartate is uncertain. Aspartate was first suggested as a neurotransmitter in the hippocampus (Nadler et al., 1976), and later in the cerebellum (Wiklund et al., 1982). The general view is that a neurotransmitter must (1) be synthesized by the neurons, (2) be taken up into synaptic vesicles and released from the nerve terminal by regulated exocytosis. Once released into the synaptic cleft, the substance must (3) act on specific receptors to give a postsynaptic response and (4) there must be an inactivation system for the substance, to ensure a high signal/noise-ratio.

Among neuroactive amino acids, glutamate, GABA, and glycine fulfill these criteria and are well established neurotransmitters.

For aspartate, points 1, 3 and 4 are well established:
(1) Aspartate can be synthesized in neurons from the TCA cycle intermediate oxaloacetate via aspartate aminotransferase (AAT; Altschuler et al., 1985; Kugler, 1987; Martinez-Rodriguez and Arenas, 1988; Schmidbaur et al., 1990). In this way aspartate can enter the "glutamate-glutamine cycle" (Gundersen et al., 1991). (3) Aspartate can excite neurons (Curtis et al., 1959), through selective activation of NMDA-type of glutamate receptors (Curras and Dingledine, 1992), making aspartate an excitatory amino acid. (4) Termination of the aspartergic signal occurs through the EAATs, which transport aspartate and glutamate with similar and high affinities from the extracellular space into intracellular compartments. A "low-affinity" uptake system for aspartate and glutamate comprising Na^+/dicarboxylate transporters has been identified in astrocytes (Holten et al., 2008). While the high-affinity transporters work at µM concentrations of aspartate and glutamate, the "low-affinity" transporters take up amino acids in the mM range, when the EAATs are saturated.

Whether aspartate is present in synaptic vesicles (point 2), is still an open question. Several studies show accumulation of aspartate in nerve terminals, the site where synaptic vesicles are located (Merighi et al., 1991; Tracey et al., 1991; van den Pol, 1991; Gundersen et al., 1998), while others show no evidence for nerve terminal accumulation of aspartate (Maxwell et al., 1990; Zhang et al., 1990; Montero, 1994; Larsson et al., 2001). Likewise, uptake studies on aspartate in synaptic vesicles have shown conflicting results (Naito and Ueda, 1983; Fykse et al., 1992). In the hippocampus, some pathways have been proposed to have aspartate-containing vesicles while other pathways show very low aspartate levels.

The VGLUTs, which are responsible for vesicular storage of glutamate, do not recognize aspartate as a transport substrate (Reimer and Edwards, 2004). The lysosomal H^+-coupled sialic acid transporter, sialin, is located throughout the brain, with especially high levels in the hippocampus (Yarovaya et al., 2005), and was suggested to transport aspartate and glutamate into synaptic vesicles in the hippocampus (Miyaji et al., 2008). Sialin is regarded a promising vesicular aspartate transporter-candidate (Miyaji et al., 2008; for review, see

Nadler, 2011), although the role of sialin in release of aspartate from intact nerve terminals has not been investigated.

There are at least four competing hypothesis as to the mechanisms for calcium-dependent aspartate depletion from the nerve terminal (figure 2): (a) Aspartate could be released from synaptic vesicles through regulated exocytosis during depolarization (in which case, aspartate would be classified as a neurotransmitter). Alternatively, (b) during depolarization, aspartate might be released through volume- regulated anion channels, presumably along with glutamate and other inorganic anions. These anion channels are not very well characterized, but several studies show their involvement in accumulation of excitatory amino acids in severe cerebral ischemia (Feustel et al., 2004; Zhang et al., 2008). (c) Glutamate, but not aspartate, could be released from synaptic vesicles during depolarization, and aspartate would then be released through the EAATs, in exchange for glutamate, or aspartate could be released through reversed transport by the EAATs during depolarization of the nerve terminal membrane. Finally, (d) when glutamate is released during depolarization, aspartate could be metabolized by AAT to give glutamate, resulting in an apparent loss of aspartate. The evidence for release, however, is massive, thus metabolism of aspartate is not likely to be the main contributor to the depletion of aspartate from nerve terminals in response to depolarization. The release mechanism for aspartate in response to depolarization was addressed in paper I and II of the present thesis.

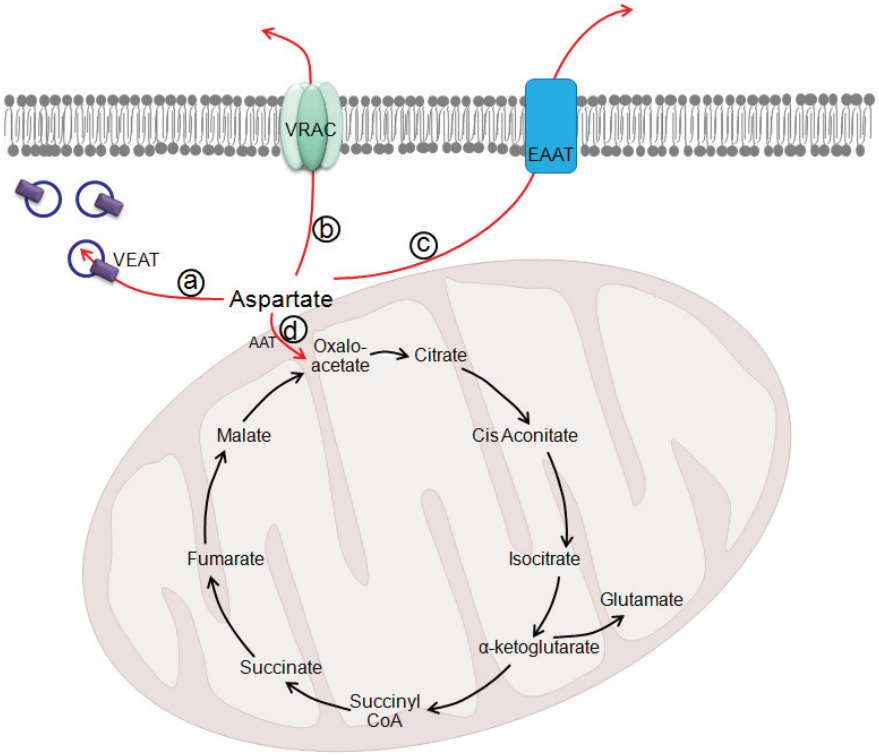

Figure 2: Suggested mechanisms underlying the depletion of aspartate from nerve terminals

a) Aspartate is taken up into vesicles by an unknown vesicular excitatory amino acid transporter (VEAT) and released through regulated exocytosis. b) Aspartate escapes through the plasma membrane volume-regulated anion channels (VRAC) or c) excitatory amino acid transporters (EAAT,) or d) aspartate is converted to glutamate via the enzyme aspartate aminotransferase (AAT).

Valproate

Valproic acid (2-propylpentanoic acid) was first synthesized in 1882 (Burton, 1882) as an organic solvent, and was used to stabilize possible anticonvulsant drugs in a laboratory test. It soon became evident that valproic acid had anti-seizure activity (Meunier et al., 1963) and the first clinical trial on Na-valproate in the treatment of

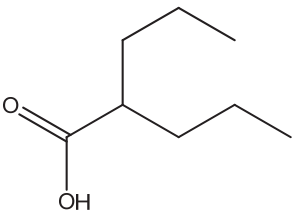

Figure 3: Valproic acid

epilepsy was reported in 1964 (Carraz et al., 1964). Valproate was released as an antiepileptic drug three years later and has been used in the treatment of epilepsy since then. Today, valproate is very well established and widely used as an antiepileptic agent, with a broad spectrum of effects against both generalized and partial seizures in adults and children. Later, other indications for valproate-treatment have been established; psychiatric disorders, migraine prophylaxis and the management of trigeminal or post-herpetic neuralgia. Although valproate is one of the most widely used drugs in neurology and psychiatry, its mechanism of action is still controversial. A regulatory effect on neurotransmission might explain some of the clinical implications for valproate, and is discussed below.

Valproate as a Tool to Investigate Synaptic Transmission

Valproate changes the brain level of aspartate and GABA.

The classic view is that epileptogenesis is caused by an imbalance between activation and inhibition of neurons, resulting from excessive glutamatergic (and possibly aspartergic) transmission, deficient GABAergic transmission, or a combination of the two. Accumulation of GABA (Godin et al., 1969; Macdonald and Bergey, 1979; Battistin et al., 1984; Löscher and Vetter, 1984a; Löscher and Vetter, 1984b; Löscher and Hörstermann, 1994) in the brain after valproate treatment is well established, and probably contributes to it´s anticonvulsant effect. In addition, valproate causes the brain levels of aspartate to decrease (Kukino and Deguchi, 1977; Schechter et al., 1978), without affecting the concentration of glutamate (Johannessen et al., 2001). The increase in GABA levels appears to be specific for the nerve terminals (Iadarola and Gale, 1979; Löscher and Vetter, 1985). The effect of valproate on the distribution of aspartate between different subcellular compartments remains unknown. If aspartate is predominantly reduced in the nerve terminals compared to, for instance, the somato-dendritic compartments, this would reflect reduced excitatory signaling and point to an additional mechanism for the anticonvulsive action of valproate. This hypothesis was tested in paper IV.

Valproate and regulation of EAATs

As mentioned above, the EAATs are important for keeping the extracellular glutamate concentration at a low level. In spite of its critical importance in pathology and normal synaptic transmission, the regulation of EAATs is poorly understood. In addition to the effects of valproate on neurotransmission, valproate is a well established histone deacetylase inhibitor, having the potential to increase gene expression (Phiel et al., 2001). Whether valproate could regulate the expression of proteins involved in transmitter signaling in the brain is largely unknown. Long-term (90 days) treatment with valproate was, however, in a previous study shown to give increased levels of EAAT2 in the hippocampus (Hassel et al., 2001). In paper IV we investigate the effect of shorter valproate treatment (2 weeks) on the EAAT2 level, glutamate uptake, and neuroprotection against excitotoxicity.

Malonate as a Neurotoxin

Malonate is a competitive inhibitor of succinate dehydrogenase (SDH), the enzyme that converts succinate to fumarate in the TCA cycle, and inhibition of SDH results in loss of ATP in the cells (Erecinska and Nelson, 1994). Malonate-induced neurotoxicity is largely excitotoxic, as these lesions can be prevented by glutamate receptor antagonists (Beal et al., 1993; Greene and Greenamyre, 1995; Ikonomidou et al., 2000). In the present work, malonate was used as a neurotoxin, to generate a lesion in rat striatum after intrastriatal injection. We demonstrate that malonate inhibit neuronal metabolism, yet leave the astrocytes capable of glutamate uptake, indicating that malonate toxicity is neuron specific.

AIM OF THE STUDIES

Determine the release mechanisms for aspartate from nerve terminals

Clarify regulatory effects of valproate on aspartergic neurotransmission

Explore the neuroprotective effect of valproate in malonate-toxicity

SUMMARY OF RESULTS

Paper I

Vesicular Release of L- and D-Aspartate from Hippocampal Nerve Terminals: Immunogold Evidence

To investigate whether release through EAATs was important for aspartate depletion from nerve terminals in response to depolarization, we exposed rat hippocampal slices to normal (3 mM) and depolarizing (55 mM) concentrations of K^+, with or without the EAAT inhibitor, threo-beta-benzyloxyaspartate (TBOA). By light- and electron microscopy analysis, we showed that the majority of aspartate depletion was EAAT-independent and that only a small fraction of aspartate depletion from hippocampal nerve terminals during K^+-induced depolarization could be blocked by TBOA. A similar release pattern was observed for the well established excitatory neurotransmitter glutamate. While L-aspartate is present at µM concentrations in the brain, endogenous D-aspartate is at trace levels. When hippocampal slices were exposed to exogenous D-aspartate before immersion-fixation of the tissue, immunogold cytochemistry showed D-aspartate uptake in terminals, where immunostaining was concentrated over synaptic vesicles as opposed to the cytoplasmic matrix. Together, these data suggest a vesicular localization of aspartate and release of aspartate through an EAAT-independent mechanism, probably exocytosis.

Paper II

Vesicular uptake and exocytosis of aspartate is independent of sialin

The release mechanism for aspartate has been debated for years, as there has been discrepancy between studies of vesicular aspartate uptake. We show that isolated synaptic vesicles were capable of accumulating aspartate, and that the vesicular uptake of radiolabeled aspartate (relative to the uptake of glutamate) varied between brain regions. The highest uptake of aspartate (18% of the uptake of glutamate) was found in the hippocampus, while the entorhinal cortex, the frontal cortex and the striatum showed uptakes of 9.6%, 10% and 12%, respectively, similar to what we observed in whole-brain

vesicles (9%). Although we demonstrate vesicular accumulation of aspartate, our results suggest that the vesicular aspartate transporter remain unidentified. Sialin has been suggested to transport aspartate and glutamate into hippocampal vesicles (Miyaji et al., 2008), but here we demonstrate that release of aspartate from nerve terminals was independent of sialin. Sialin knock-out mice and wild-types showed the same ability to deplete aspartate from the nerve terminals in response to depolarization. We further investigated the importance of two suggested non-vesicular release mechanism for aspartate; volume-regulated anion channels (VRACs) and excitatory amino acid transporters (EAATs), but found no evidence for release through these mechanisms. We confirmed that the depletion of aspartate from nerve terminals was strictly calcium dependent, as it could be completely inhibited by replacing extracellular calcium with magnesium. Furthermore, KB-R7943, which inhibits calcium influx both through the reversed Na^{+}/Ca^{2+} exchanger, transient receptor potential channels, and NMDA receptors (Sobolevsky and Khodorov, 1999; Arakawa et al., 2000), inhibited both aspartate and glutamate depletion. We conclude that aspartate is accumulated in synaptic vesicles and released through exocytosis after vesicular accumulation by a yet unidentified transporter.

Paper III

Valproate causes reduction of the excitatory amino acid aspartate in nerve terminals

The antiepileptic and mood stabilizing drug, valproate, causes the brain level of the excitatory transmitter aspartate to decrease. The effect of valproate on the distribution of aspartate between different subcellular neuronal compartments, however, remains unknown. Here we show that valproate treatment caused decreased aspartate levels in both excitatory and inhibitory nerve terminals. The decrease occurred selectively in nerve terminals, as the aspartate level in stem dendrites was largely unchanged. Glutamate and GABA in excitatory and inhibitory nerve terminals, respectively, showed only minor changes after valproate treatment. The most pronounced effect, thus, was that on aspartate; A 64% reduction in excitatory terminals and a 62% reduction in inhibitory

terminals. Our data point to a potentially important clinical mechanism for valproate; reduced aspartergic neurotransmission.

Paper IV

Valproate is neuroprotective against malonate toxicity in rat striatum: an association with augmentation of high-affinity glutamate uptake

Valproate has neuroprotective properties, and several mechanisms have been proposed to underlie this effect. We treated rats with valproate for 14 days (300 mg/kg twice daily) before malonate (1.5 µmol; 1 M) was injected into their right striatum. We found that valproate-treated animals developed smaller lesions than control animals in response to malonate toxicity. The lesions were due to malonate toxicity and not hyperosmolarity, since injection of NaCl that was equiosmolar with 1 M malonate, caused lesions that were about 20 times smaller than the lesions caused by malonate. Injection of physiologic saline did not cause lesions. By microdialysis, we showed that valproate pre-treatment of rats reduced extracellular accumulation of glutamate and aspartate in response to malonate toxicity in the striatum. This effect paralleled an increase in the striatal level of the excitatory amino acid transporter EAAT2, and an augmentation of high-affinity glutamate uptake into striatal proteoliposomes. Malonate injection caused a reduction in striatal adenosine triphosphate (ATP) content, however our data indicate that malonate did not inhibit the glial ATP production, which is necessary for uptake of glutamate. Western blot analysis showed that the striatal levels of HSP-70 and fos were reduced, and the levels of bcl-2 and phosphorylated extracellular signal-regulated kinase (p-ERK) remained unaffected. Histone acetylation was increased by valproate treatment. The results suggested that augmentation of excitatory amino acid uptake was an important contributor to valproate-mediated neuroprotection in the striatum. The results further suggest that increased uptake of excitatory amino acids from the extracellular fluid may be a mechanism of action of valproate as an antiepileptic drug.

DISCUSSION

Is Aspartate a Neurotransmitter in the Brain?

Aspartate fulfills most criteria normally required to be classified as a neurotransmitter, but the release mechanism of aspartate has been debated for 30-40 years and still remains unresolved. The major point of discussion has been whether aspartate is packed into synaptic vesicles and released from nerve terminals by exocytosis, or if the release occurs primarily through plasma membrane transporter(s) or channel(s).

Immunostaining for aspartate is found in the nerve terminals, where it is higher over synaptic vesicles than over the cytoplasmic matrix (Gundersen et al., 1998). These data clearly demonstrate the presence of aspartate in the terminals, and suggest accumulation of aspartate in synaptic vesicles, however, they are not conclusive. Theoretically, during the fixation process, amino acids in close proximity to protein rich organelles, like synaptic vesicles, have a better chance of being fixed in the tissue than amino acids in less protein-rich areas, like the cytosol. However, while the putative neurotransmitters glutamate and aspartate showed significant association with synaptic vesicles, the labeling for the non-transmitter amino acids glutamine and taurine, was equally distributed between the vesicle clusters and the cytoplasmic matrix (Gundersen et al., 1998). Thus, intra-vesicular localization of aspartate is likely, but binding of cytosolic aspartate to the vesicular surface could also explain the association of aspartate with synaptic vesicles. These arguments also apply to the accumulation of exogenous D-aspartate reported in paper I.

In paper II, we show that aspartate is taken up into synaptic vesicles. This has been an open question until now, as some studies have demonstrated vesicular uptake and localization of aspartate (Naito and Ueda, 1983; Fleck et al., 2001; D'Aniello et al., 2011) while others have found vesicular accumulation of aspartate to be negligible (Maycox et al., 1988; Fykse et al., 1992). This discrepancy is not easily understood, as authors have used similar methods, but arrives at highly different answers (see for instance Naito and Ueda, 1983; Fykse et al., 1992). Both D'Aniello and co-workers (2011) and Fleck and co-workers (2001) found that aspartate levels in synaptic vesicles were similar to glutamate levels, however the former group detected a higher level of aspartate in whole brain

homogenate (aspartate:glutamate ratio = 0.8) than reported by most other authors (a ratio of 0.2-0.3; Paper IV; Liebschutz et al., 1977; Fonnum et al., 1981; Alvestad et al., 2008; Kokras et al., 2009). The latter study did not exclude the possibility for enzymatic conversion of [^{3}H]L-aspartate (and possibly [^{3}H]D-aspartate, via D-aspartate racemase or D-aspartate oxidase) to [^{3}H]-glutamate in the synaptosomes prior to vesicular uptake.

The aspartate uptake reported in paper II, 9.1% (of the glutamate uptake), is lower than indicated by the studies of D'Aniello et al. (2011) and Fleck et al. (2001), but consistent with the value obtained by Naito and Ueda (1983). Our experiments eliminate the possibility of unspecific temperature-dependent binding to the vesicles, as our negative controls (the radioactivity of which were subtracted from the radioactivity measured in the samples, to calculate the actual uptake) were treated identically to the samples, except that ATP was omitted from the negatives. Thus, we measure only the ATP dependent uptake of aspartate in the vesicles.

The principal step in vesicular release, fusion with the plasma membrane, is triggered by a rise in intracellular calcium, and experiments with different brain preparations from different brain regions show that the release of aspartate in response to depolarization is highly calcium -dependent (Girault et al., 1986; Burke and Nadler, 1988; Szerb, 1988; Paulsen and Fonnum, 1989; Gundersen et al., 1991; Roisin et al., 1991; Fleck et al., 1993; Zhou et al., 1995; Gundersen et al., 1998; Bradford and Nadler, 2004; Zappettini et al., 2010), although some studies failed to demonstrate calcium dependency of the aspartate release (Levi et al., 1982; Wilkinson and Nicholls, 1989; McMahon and Nicholls, 1990). The depletion of aspartate described in this thesis is strictly dependent on extracellular calcium, as reducing calcium in the incubation medium to 0.1 mM (magnesium elevated to 10 mM) totally prevented aspartate depletion in response to depolarization (paper II). The demonstration of calcium dependent release is an indicative, but not conclusive, confirmation of exocytosis.

The action of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins are essential for exocytotic release of neurotransmitters, and dependency of SNARE proteins is generally regarded as a definite test for exocytosis. Clostridal bacteria synthesize a series of neurotoxins that inhibit priming of synaptic

vesicles during the process of exocytosis; tetanus toxin (TTX) and botulinum toxins (BoNT) B, D, F, G and H which cleave the vesicular SNARE synaptobrevin, BoNT/A and E, which specifically cleave SNAP-25, and BoNT/C, which cleave both SNAP-25 and syntaxin. Studies on the effect of botulinum and tetanus toxin on aspartate release show conflicting results, as sensitivity towards clostridal toxins has been demonstrated in hippocampal slices (Gundersen et al., 1991; 1998) and some studies on synaptosomes (McMahon et al., 1992; Wang and Nadler, 2007; Cavallero et al., 2009), but not in another (Bradford and Nadler, 2004). Clostridal toxins, consist of a light chain and a heavy chain. Cleavage of the SNARE proteins by these toxins, is dependent on internalization of the proteolytic light chain. This occurs through endocytosis during vesicle recycling, and requires binding of the heavy chain to vesicular proteins expressed on the plasma membrane. Some researchers believe that the release of aspartate occurs through exocytosis of vesicles at ectopic locations (for discussion, see Nadler, 2011). As vesicle recycling, and thus the internalization of clostridal toxins mainly takes place near the active zone, the intracellular concentration of the toxins are likely to be highest in this area. Thus, the concentration of the toxin at the locations for aspartate release might not be high enough to inhibit exocytosis. Although some conflicting results exist regarding the sensitivity or aspartate release to clostridal toxins, most release data point to a clostridium toxin-sensitive, most likely vesicular, release mechanism for aspartate.

The major objection to the concept of aspartergic vesicular release is that no vesicular aspartate transporter has been identified. The lysosomal H^+-coupled sialic acid transporter, sialin, was suggested to transport aspartate and glutamate into synaptic vesicles in the hippocampus (Miyaji et al., 2008). Sialin is located throughout the brain, with especially high levels in the hippocampus (Yarovaya et al., 2005) and has been regarded a vesicular aspartate transporter-candidate (Miyaji et al., 2008; for review, see Nadler, 2011), even though the role of sialin in release of aspartate from intact nerve terminals has not been investigated. In paper II we show that aspartate labeling under basal conditions and aspartate depletion from nerve terminals in response to depolarization is equal in slices from sialin knock-out mice and controls, indicating that sialin is not important for vesicular accumulation and release of aspartate. These results suggest that sialin is either not present in sufficient amounts in the vesicular membrane, or that sialin does not transport

aspartate under physiological conditions. In the study by Miyaji and co-workers (2008), the authors report to have detected sialin in the hippocampal P2 fraction. According to standard nomenclature, the P2 fraction refers to a crude synaptosome fraction (see for instance Kadota and Kadota, 1973; Huttner et al., 1983), which contains mitochondria and probably lysosomal membranes. Thus, the localization of sialin in synaptic vesicles is still uncertain. The assumption that sialin is not a constituent of synaptic vesicles is supported by Takamori and co-workers (2006) who did not detect sialin in a proteomics study of synaptic vesicles. According to Miyaji and co-workers (2008), the affinity of sialin is similar for aspartate and glutamate, and higher than the affinity of the VGLUTs for glutamate. In neurons, the intracellular concentration of aspartate is about 1:5 of the glutamate concentration (Nadler et al., 1976). If sialin was present in sufficient amounts to be important for vesicular aspartate accumulation, it would probably also be important for the uptake of glutamate. This is contradicted by data from VGLUT knock-out animals (Fremeau, Jr. et al., 2004). Altogether, the evidence suggests that sialin is not important for the uptake and release of aspartate from nerve terminals.

Alternative Release Mechanisms for Aspartate

Reversal of EAATs (Szatkowski et al., 1990) has been confirmed a major contributor to the high extracellular levels of EAAs, including aspartate, in severe brain ischemia (Phillis et al., 1998; Seki et al., 1999). However, in our hippocampal slices we show that the EAATs are not a major release mechanism for aspartate during neuronal membrane depolarization (papers I and II), since inhibition of EAATs with TBOA did not prevent depletion of aspartate.

Release of aspartate and glutamate through volume-regulated anion channels (VRAC) has been shown to be an important contributor to extracellular accumulation of these excitatory amino acids in ischemia (Feustel et al., 2004; Zhang et al., 2008) and spreading depression (Basarsky et al., 1999). As *in vivo* studies do not differentiate between amino acids released from neurons and astrocytes, the cellular origin of the excess excitatory amino acids during these pathological conditions is not known, although the effect of VRAC

inhibition on release of EAAs has been confirmed in cultured astrocytes (Rutledge et al., 1998). The molecular identity of VRAC is unknown (for discussion, see Eggermont et al., 2001; Hoffmann et al., 2009), and it is not known whether the concept of VRAC refers to one single channel or to a whole family of related anion channels. Consequently, antibodies against these channels have not been developed and the localization of these channels can only be based on physiological experiments. Our data indicate that in neurons, at least in the presynaptic terminals, VRACs are not a major release mechanism for neither aspartate nor glutamate, as inhibition of VRAC by NPPB, did not alter the content of these amino acids in synaptic terminals. The concentration of NPPB used in this study (100μM) has been shown to almost completely inhibit VRAC-mediated Cl^--currents (Sagheddu et al., 2010; Zhang et al., 2011). Our findings are consistent with studies demonstrating that, while astrocytes are likely to undergo rapid volume regulation through swelling in response to acute pathological states (Van Harreveld, 1966; Kimelberg et al., 2000; Mongin and Kimelberg, 2005; Risher et al., 2009), neuronal volumes (Andrew et al., 2007) appear resistant to changes in external osmolarity. The neuronal volume stability and lack of fast volume regulation is likely due to the lack of aquaporins on the neuronal plasma membrane, giving neuronal membranes low water permeability. Whether neurons express VRAC is not known (but see Zhang et al., 2011).

It should be mentioned that NPPB inhibits connexin Cx43 (Ye et al., 2009) and monocarboxylate transporters (MCTs) (Carpenter and Halestrap, 1994; Rinholm et al., 2011), at the same concentrations that block VRAC. Cx43 is primarily expressed on astrocytes, where it exists in half of a gap junction (hemichannel) (Bennett et al., 2003; Ye et al., 2003; Spray et al., 2006) that are capable of releasing molecules less than 1kD, including amino acids. To my knowledge, the localization of connexins in the nerve terminal membranes has not been reported, and the inhibitory effect of NPPB on Cx43 is therefore not likely to affect the nerve terminal content of aspartate and glutamate directly. Since the MCTs do not transport amino acids, and have not been reported as constituents of the plasma membrane of nerve terminals, a direct effect of MCT inhibition on release of aspartate and glutamate is not likely. However, inhibition of mitochondrial MCTs (Butz et al., 2004; Hashimoto et al., 2008), which transport pyruvate and lactate

across the inner mitochondrial membrane, would lead to functional hypoglycemia and subsequently to increased aspartate levels (Gundersen et al., 1998). In parallel analyses we demonstrate that aspartate accumulates in astrocytes in response to NPPB exposure (data not shown), while the aspartate level in nerve terminals (paper II) is unchanged. This finding suggests a selective action of NPPB on astrocytes.

Taken together, our data (paper I and II) do not support the notion of VRACs and EAATs as important release mechanisms for aspartate from nerve terminals under physiological conditions, leaving exocytosis as the most likely release mechanism.

Aspartate in Inhibitory Synapses

Both in perfusion-fixed rat hippocampus and in mouse and rat brain slices, the labeling density of aspartate in inhibitory terminals was approximately twice that in excitatory terminals (papers II and III). This is in agreement with several studies showing higher aspartate levels in GABAergic neurons than in glutamatergic neurons (Storm-Mathisen et al., 1986; Hassel et al., 1995; Gundersen et al., 2001a) and probably has a metabolic explanation: The rate-limiting enzymes of the oxidative metabolism are pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Lai et al., 1977; Morland et al., 2007). In GABAergic neurons the GABA-shunt (Figure 4) can circumvent the step catalyzed by α-ketoglutarate, facilitating the flux through to oxaloacetate. The conversion of oxaloacetate to citrate, however, is dependent of acetyl-CoA formed by pyruvate dehydrogenase. Thus oxaloacetate, and subsequently aspartate, accumulates in these neurons. What the physiological functions of aspartate release from inhibitory terminals might be, is an intriguing question. At GABAergic synapses, aspartate is probably the main agonist acting on the NMDA-receptors, which have been found at high densities along the post-synaptic membranes of these synapses (Gundersen et al., 2004).

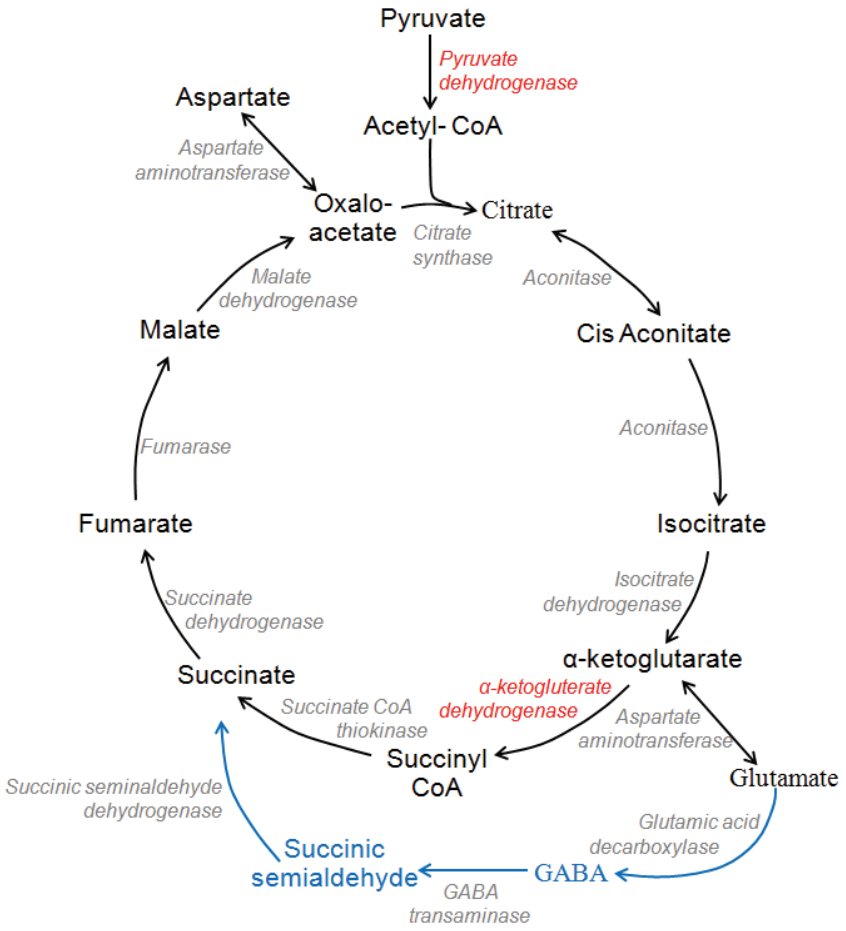

Figure 4: TCA cyclewith the GABA shunt

TCA cycle (black) and GABA shunt (blue). The rate limiting enzymes are in red

Valproate

Dosage and Route of Administration

Valproic acid is almost fully dissociated at physiological pH, giving it a high water solubility and a low volume of distribution compared to most anticonvulsant drugs (Löscher and Frey, 1984). A steady-state situation, where the concentration of valproate in cerebrospinal fluid (CSF) equals about 20 % of the serum concentration, is rapidly reached,

as the valproate level in brain and CSF peak at approximately the same time as the plasma concentration (Löscher and Esenwein, 1978). This predictable access of valproate to the brain justifies systemic administration of valproate, as used in this thesis. The elimination of valproate is mainly through biotransformation (reviewed in Baillie and Sheffels, 1995). While the half-life of valproate in humans is reported as 9-18 hours, the degradation in rats is much faster (elimination half-life of 2-5; non-linear kinetics; Löscher, 1999). This explains why higher doses of valproate are required in rats to obtain plasma concentrations within the therapeutic window used for humans. The dose used in this thesis, 300mg/kg, twice daily, is within the range use in other *in vivo* studies (Kukino and Deguchi, 1977; Chapman et al., 1982; Löscher and Hörstermann, 1994; Hassel et al., 2001; Johannessen et al., 2001)

Valproate and Brain Energy Metabolism: Effects on GABA and Aspartate

Because of the wide spectrum of actions against different types of seizures and neuro-psychiatric disorders, valproate is suggested to act by a combination of different mechanisms.

A bulk increase in GABA (Godin et al., 1969) accompanied by a reduction in aspartate (Chapman et al., 1982; Löscher and Hörstermann, 1994) has been suggested as an important anti-epileptic mechanism for valproate. The increase in GABA is believed to occur predominantly in inhibitory nerve terminals (Iadarola and Gale, 1979; Löscher and Vetter, 1985). In paper II, we show that the decrease in aspartate occurs selectively in nerve terminals. In excitatory nerve terminals, only aspartate and not glutamate, was significantly reduced by valproate treatment. This is in line with biochemical studies showing that valproate selectively inhibits the release of aspartate in preference to glutamate (Crowder and Bradford, 1987; Biggs et al., 1992). Similarly, in inhibitory terminals, the decrease in aspartate was much more pronounced than the increase in GABA. Valproate inhibits the intramitochondrial enzyme α-ketoglutarate dehydrogenase (Johannessen et al., 2001), which leads to reduced concentration of oxaloacetate and, thereby of aspartate, which is formed from oxaloacetate via the aspartate aminotransferase reaction. The selective effect in nerve terminals might be due to a higher turnover of transmitter amino acids in nerve terminals than in somato-dendritic

compartments (Hassel et al., 1997). Reduced aspartergic neurotransmission might be an additional antiepileptic effect of valproate.

Inhibiton of voltage-gated sodium channels?

Another proposed mechanism of action for valproate is inhibition of sustained repetitive firing (SRF) of neurons, due to inhibition of voltage-gated sodium channels (Slater and Johnston, 1978; McLean and Macdonald, 1986; Vreugdenhil et al., 1998) or enhancement of potassium channel function involved in action potential repolarization (Morre et al., 1984; Franceschetti et al., 1986). However, a direct effect of valproate on voltage-gated sodium channels has been questioned (Löscher, 1993; Albus and Williamson, 1998), and the effect on potassium channels has only been demonstrated at supra therapeutically doses of valproate. Consequently, inhibition of SRF is has been disregarded as a clinically important mechanism of action (Löscher, 1999; Johannessen et al., 2001), further underscoring the importance of discovering other mechanisms of action, as for instance that of aspartate as discussed above.

Malonate as a Model for Energy Depletion; Selective Blockage of Neuronal Energy Metabolism

Malonate is a competitive inhibitor of succinate dehydrogenase (SDH), a key enzyme in both the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation, and thus inhibition of SDH results in loss of ATP in the cells (Erecinska and Nelson, 1994). The brain lesions caused by injection of malonate can be prevented by glutamate receptor antagonists (Beal et al., 1993; Greene and Greenamyre, 1995; Ikonomidou et al., 2000), indicating that excitotoxicity is a key mechanism. Inhibition of neuronal metabolism impairs the neurons' ability to maintain resting membrane potential. The resultant depolarization may relieve the voltage-dependent magnesium block of the NMDA receptor, allowing for easier receptor activation and greater flux through the receptor's ion channel. The resultant excessive inward flux of predominantly Ca^{2+}, but also Na^{+}, might lead to neuronal death. Thus, excitotoxicity after metabolic inhibition seems to be mainly

through excessive NMDA-receptor activation, thus the increase in extracellular glutamate that we and others (Messam et al., 1995) find after intrastriatal malonate injection is likely to play an important role for the neurotoxicity of malonate.

To understand how valproate can be neuroprotective against malonate toxicity, selectivity of malonate action on neurons over astrocytes is an important aspect. Several findings suggest that malonate inhibits the energy metabolism in neurons but not in astrocytes: First of all, malonate is selectively taken up into neurons, as the specific activity of glutamate is higher that that of glutamine after injection of radiolabeled malonate (Koeppen et al., 1978; Mitzen and Koeppen, 1984; Hassel et al., 2002). Second, malonate toxicity is characterized by inhibition of glutamate synthesis from (radiolabeled) glucose (Paper IV), a predominantly neuronal reaction (Storm-Mathisen et al., 1983; Hassel et al., 1997). Third, ATP-dependent formation of glutamine from radiolabeled glucose (e.g. Powers and Riordan, 1975), a strictly glial reaction in the brain (Martinez-Hernandez et al., 1977), was not reduced in response to malonate poisoning. Radiolabeled glutamate injected into malonate-treated striatum was converted to glutamine (paper IV), strongly suggesting that the astrocytes were capable of glutamate uptake and glutamine synthetase activity, two ATP dependent processes, even in the presences of malonate. Together these findings indicate that, although neuronal energy metabolism was inhibited, astrocytes could generate energy to support glutamate transport during malonate exposure. Thus, the valproate-induced increase in EAAT2 (paper IV) most probably led to increased glutamate uptake into astrocytes *in vivo* and reduced excitotoxic stress on the neurons, and therefore contribute to the neuroprotective effect.

METHODOLOGICAL CONSIDERATIONS

Advantages and Disadvantages of Using *in vitro* Preparations

In this thesis I have used some *in vitro* models: hippocampal slices, isolated synaptic vesicles and proteoliposomes. *In vitro* models are suitable for studies of detailed molecular mechanisms, which are not easily investigated in whole animals. *In vitro* preparations can easily be manipulated with, without the ethical consideration of animal welfare, which is necessary when doing *in vivo* experiments. Thus, they can be invaluable models and replacements for *in vivo* studies. Although *in vitro* models in many cases are important test systems, extrapolation to the *in vivo* situation should always be done with caution. Differences between *in vitro* and *in vivo* results can reflect for instance endocrine- or paracrine factors, immune responses, direct communication with other cells, and availability of nutrients and oxygen.

For papers I and II we used hippocampal slice preparation as an *in vitro* model for hippocampal function. The main advantage with this model is that one can study the effects of alterations in the external milieu on signal transduction systems, as the cell-to-cell interactions remain and can be studied. As for any *in vitro* model, hippocampal slices have the disadvantage of being an isolated brain area removed from the circuitry of the intact brain. Also, some morphological changes are known to happen during slice incubation; increased swelling or condensation of some brain compartments and retraction of the finer astrocyttic processes (Fiala et al., 2003). However, hippocampal slices form a well defined and highly characterized test system, and has been invaluable for the studies presented in papers I and II of this thesis.

Synaptic Vesicles

Isolation of synaptic vesicles is a commonly used method to study the uptake ability of these organelles. When synaptic vesicles are isolated by simple differential centrifugations, the resulting vesicular fraction will, to a variable degree, be contaminated with other

cellular compartments. Contamination by myelin and cell membranes in crude synaptic vesicle fraction might be reduced by mixing the supernatant gained after the first low-speed centrifugation step with sucrose, final concentration 0.8M (Mariussen and Fonnum, 2001). Further purification of the synaptic vesicles may be performed with controlled-pore glass bead chromatography (Nagy et al., 1976; Huttner et al., 1983;Jahn et al., 1985) or by immunoprecipitation with antibodies against obligatory synaptic proteins like synaptobrevin or synaptophysin (Burger et al., 1989; Burré et al., 2007). However, all these purification steps have the disadvantage that a considerable amount of synaptic vesicles is lost. For the experiments included in this thesis, synaptic vesicles from small brain regions were used and the amount of vesicles gained from each of these structures was limited, thus the crude synaptosomal fraction was used without further purifications. The observed uptake of aspartate and glutamate (paper II) can not be ascribed to plasma membrane EAATs in contaminating membrane-enclosed structures, as uptake via the EAATs require Na+ (Storck et al., 1992; Pines et al., 1992; Kanai and Hediger, 1992; Tanaka, 1993; Arriza et al., 1997), which was obmitted during the isolation- or incubation steps.

Antibody Testing

The use of antibodies for immunhistochemistry requires a great deal of caution, as cross-reactivity and unspecific labelling might occur. This matter has been exhaustively discussed by Holmseth et al. (2005). In a recent paper (Larsson et al., 2011) we highlighted the importance of testing antibodies in the same tissue as the analysis were performed, as cross-reactivity can be highly tissue-specific. The antibodies used in this thesis have been specificity-tested by Western blotting (for antibodies against proteins) and shown to give one principal band at the appropriate molecular weights. The antibodies against amino acids have been extensively characterized (Gundersen et al., 1993; 1998; 2001a; 2001b) yet, for each experiment, sandwiches of different amino acids conjugated to brain proteins (Ottersen et al., 1990) were included to verify that the antibodies labeled only the amino acid against which they were raised.

CONCLUDING REMARKS

We have studied the mechanism underlying release of aspartate from nerve terminals and the effect of an antiepileptic drug, valproate, on cerebral levels of aspartate and glutamate.

In particular, we have answered the following questions:

How do synaptic terminals release aspartate?
We have shown that aspartate is taken up into synaptic vesicles and presumably released by regulated exocytosis. Alternative release mechanisms, plasma membrane EAATs and VRACs have been excluded as important contributors to aspartate release under non-pathological conditions (papers I and II).

Is sialin the principal vesicular aspartate transporter?
We used sialin knock-out mice and wild-types to show that sialin is not required for depletion of aspartate from nerve terminals in response to depolarization. Thus, vesicular accumulation of aspartate must be highly independent of sialin.

Can the antiepileptic drug, valproate, reduce aspartergic neurotransmission?
Acute treatment of rats with valproate, resulted in decreased levels of aspartate in excitatory and inhibitory nerve terminals, but not in stem dendrites, indicating an effect on the transmitter pool of aspartate. These results point to a possibly important clinical effect of valproate: reduced aspartergic neurotransmission.

Is valproate neuroprotective against excitotoxicity?
In response to two weeks of valproate treatment, the level of EAAT2 was increased, and the extracellular accumulation of aspartate and glutamate in response to a focal injection of a neurotoxin, malonate, was reduced. Consequently, valproate treated rats developed smaller lesions than the control animals did, indicating a neuroprotective effect of valproate treatment *in vivo*.

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I

Vesicular Release of L- and D-Aspartate from Hippocampal Nerve Terminals: Immunogold Evidence

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Abstract: Glutamate is established as the most important excitatory transmitter in the brain. The transmitter status of aspartate is debated. There is evidence that aspartate is released from nerve terminals by exocytosis. However, release through excitatory amino acid transporters (EAATs) could be an alternative mechanism. We further investigated this by use of light and quantitative electron microscopic immunocytochemistry. The nerve terminal localisation of aspartate was compared to that of glutamate using antibodies specifically recognising the amino acids. Rat hippocampal slices were incubated under normal (3 mM) and depolarising (55 mM) concentrations of K^+ with and without the excitatory amino acid transporter inhibitor threo-beta-benzyloxyaspartate (TBOA). If aspartate is released either through reversal of the EAATs or through exchange with synaptically released glutamate, we would expect that TBOA would block the depolarisation induced release of aspartate. We found, however, that there was a substantial depletion of aspartate, as well as of glutamate, from hippocampal nerve terminals during K^+ induced depolarisation in the presence of TBOA. The possibility that aspartate is released through exocytosis from synaptic vesicles was further investigated by the use of a D-aspartate uptake assay, including exposure of the slices to exogenous D-aspartate and the use of D-aspartate immunogold cytochemistry to localise D-aspartate in the fixed tissue. We found that D-aspartate taken up into the terminals was concentrated in synaptic vesicles as opposed to in the cytoplasmic matrix. This is in line with the presence in synaptic vesicles of the recently identified vesicular transporter for aspartate.

Keywords: Synaptic vesicles, immunocytochemistry, amino acids, electron microscopy, transport.

The release of glutamate at synapses in the brain is well studied. Glutamate is released upon depolarisation of the presynaptic nerve terminal, leading to opening of voltage gated Ca^{2+} channels and Ca^{2+}-dependent fusion of glutamate filled synaptic vesicles with the plasma membrane. The mechanism of release of L-aspartate at central synapses remains less defined. L-aspartate has been shown to be released in a Ca^{2+} and clostridium toxin sensitive manner from various *in vitro* brain preparations (e.g. [1-7]). Strongly in favour of an exocytotic release mechanism are our previous immunocytochemical results showing that K^+ induced depolarisation elicited depletion of L-aspartate from nerve endings, which could be inhibited by low extracellular Ca^{2+}-concentrations and tetanus toxin [8-10]. In addition, D-aspartate has been shown to be subject to exocytotic release both from cultured neurons [11] synaptosomes [12] and intact brain tissue [13]. Taken together, these results indicate that synaptic vesicles harbour a transporter for L- and/or D-aspartate (see [14]). This is in good agreement with a very recent study showing that hippocampal synaptic vesicles contain a vesicular excitatory amino acid transporter (VEAT) for aspartate and glutamate [15]. It was shown that sialin, a H^+/sialic acid co-transporter belonging to the SLC17A5 group of Na^+/phosphate transporters, could carry also aspartate and glutamate into synaptic vesicles [15].

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There is ample evidence that excitatory nerve endings in the brain contain functional plasma membrane excitatory amino acid transporters (EAATs) [16-20]. The previous aspartate release data do not clearly distinguish between L-aspartate release *via* exocytosis and *via* these EAATs, which could release aspartate either through reversal of the transporter or through heteroexchange with glutamate released from synaptic vesicles. Although some biochemical studies have given evidence that release through the EAATs does not play a pivotal role in the release of aspartate [12, 21], this has not been directly studied with high resolution at the morphological level. In order to investigate the proportion of L-aspartate release which is mediated through the EAATs, we incubated hippocampal slices with the non-transportable glutamate transporter inhibitor (2S,3S)-3-[3-(4-methoxybenzoylamino)benzyloxy]aspartate (PMB-TBOA) during K^+-induced depolarisation. By using light- and electron microscopic immunocytochemistry we compared the depletion of L-aspartate from excitatory nerve terminals with the depletion of glutamate. To shed additional light on the vesicular release of aspartate we exposed the slices to exogenous D-aspartate and used a quantitative D-aspartate immunogold method [16-18, 22] to analyse the synaptic vesicle content of D-aspartate that had been taken up into excitatory nerve terminals.

MATERIALS AND METHODOLOGY

Hippocapal Slice Incubation

Hippocampal slice experiments were performed as previously described [8, 9, 18]. In brief, in each experiment we

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harvested a brain from a decapitated Wistar rat (~200 g). Then the hippocampi were dissected out and cut in 0.3 mm thick slices. The slices were preincubated in oxygenated Krebs' solution (126 mM NaCl, 3 mM KCl, 10 mM sodium phosphate buffer (pH 7.4), 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 10 mM glucose) for 1 h at 30 º C. The slices, which were subjected to EAAT inhibition, were transferred to a Krebs' solution containing 30 μM PMB-TBOA [18] during the last 15 minutes of the preincubation. Then the slices were incubated for 1 h at 30 °C in Krebs' solution under physiological (3 mM) and depolarising (55 mM) concentrations of K^+ with and without the EAAT-blocker PMB-TBOA (30 μM) (6 slice experiments). To characterise the localization of D-aspartate taken up in the nerve terminals, after preincubation some slices were incubated for 15 minutes in Krebs' solution at 3 mM K^+ with and without 50 μM D-aspartate (3 slice experiments). After incubation the slices were fixed in 2.5% glutaraldehyde and 1% formaldehyde for 1 h in room temperature (about 20°C). The slices were stored in the fixative at 4°C until sectioning and embedding.

Light Microscopic Immunocytochemistry

The hippocampal slices were cryo-protected in sucrose (30%) before 20 μm thick sections were cut on a freezing microtome. Then the sections were subjected to immunocytochemistry according to a three layer immunoperoxidase method. After incubation with primary antibodies (no 435 L-aspartate (1:5000) and no 607 L-glutamate (1:5000)) overnight at room temperature (about 20°C), the sections were treated with biotinylated donkey anti-rabbit Ig (Amersham, Little Chalfont Buckinghamshire, UK) and with streptavidin-biotinylated horseraddish peroxidase (Amersham, Little Chalfont Buckinghamshire, UK), before the antigen-antibody binding was visualized with hydrogen peroxide/di-amino-benzidine.

Electron Microscopic Immunocytochemistry

The slices were embedded in Lowicryl HM20 as previously described [18, 22-24]. After the embedding ultrathin sections (80-100 nm) were cut perpendicular to the surface of the slices (so that immunogold analysis could be made at definite distances from the slice surface) and labelled with the 482 D-aspartate (1:300), 607 L-glutamate (1:5000) or 435 L-aspartate (1:100) antisera. The primary antibodies were visualised with 15 nm gold particles coupled to secondary anti-rabbit antibodies. The sections were studied in a Tecnai 12 or a Philips CM10 electron microscope. Electron micrographs were randomly taken from the CA1 stratum radiatum and the inner dentate molecular layer. The number of L-aspartate, D-aspartate and glutamate gold particles were counted in nerve terminals making asymmetric synapses on dendritic spines (excitatory synapses). The area of the nerve terminals (the area of mitochondria was not included) was estimated by point counting using an overlay screen [23] and the gold particle densities were calculated. The results were statistically evaluated by a non-parametric Mann-Whitney U test (Statistica).

For the analysis of D-aspartate in synaptic vesicles gold particles signalling D-aspartate and grid points for area determination were recorded over synaptic vesicles and cytoplasmic matrix (areas free of vesicles and mitochondria) in excitatory nerve terminals. Synaptic vesicles were identified as round, clear membrane bounded structures with a diameter ranging from 20 to 80 nm [9]. As the lateral resolution of the immunogold method is about 30-40 nm [25, 26], which is similar to the diameter of the synaptic vesicles and to the section thickness, the method cannot localize a gold particle to a single vesicle. To by pass this problem, gold particles were ascribed to synaptic vesicles if the particle centres were within a 30 nm distance from the outer border of the vesicles. To correct for the contamination of the vesicle labelling with the cytoplasmic matrix labelling, the gold particle densities recorded over cytoplasmic matrix were subtracted from the gold particle densities recorded for the vesicles. The areas of the profiles included in the analysis were determined by point counting using an overlay screen [23]. The densities of D-aspartate immunogold particles (average number of gold particles/μm^2) in the different tissue profiles were calculated and the results statistically evaluated by a non-parametric test (Mann-Whitney-U, Statistica).

Specificity of the Antibodies

The rabbit no 607 L-glutamate, no 435 L-aspartate and no 482 D-aspartate antisera have been previously characterized [9, 10, 16, 27]. To avoid possible cross-reactivities the L-glutamate, L-aspartate and D-aspartate antisera were used with the addition of 0.2 mM complexes of glutaraldehyde/formaldehyde treated L-aspartate plus glutamine and L-asparagine, L-glutamate plus GABA and L-glutamate plus L-aspartate, respectively. As a specificity control for each immunoperoxidase incubation, spots of conjugates of different amino acids linked to brain macromolecules by glutaraldehyde/formaldehyde were placed on cellulose nitrate-acetate filters and incubated along with the tissue sections [8, 9]. For the immunogold experiments conjugates were made according to [28]. The concentration of the fixed amino acids in the embedded conjugate clumps is about 100 mM [28]. These test systems showed that the primary antibodies only labelled the conjugate containing the amino acid against which the antibodies were raised. Furthermore, the glutamate, L-aspartate and D-aspartate immunoreactivities of tissue and test sections were blocked by adding 0.3 mM of aldehyde treated glutamate, L-aspartate or D-aspartate, respectively, to the antisera. The relationship between the density of L-aspartate, D-aspartate and glutamate immunogold particles and the concentration of fixed glutamate in the tissue was linear, as assessed by simultaneously processed test sections with conjugates containing known concentrations of glutamate, L-aspartate and D-aspartate [16, 29].

RESULTS

Effects of Membrane Depolarisation and TBOA

We have previously shown that K^+ induced membrane depolarisation causes aspartate, in the same manner as glutamate, to be depleted from excitatory nerve endings in a Ca^{2+}-dependent and tetanus toxin sensitive manner [8,9]. The question is whether such a depletion of aspartate could be caused by heteroexchange through the EAATs with exocytosed glutamate. An ideal tool for investigating this would be to use a non-transportable blocker of the EAATs during membrane stimulation, such as threo-beta-benzyloxy-aspartate (TBOA) [30]. In a recent study we showed that 30 μM PMB-TBOA, which is a potent blocker of EAAT1-3 [31], totally inhibits D-aspartate transport in exci-tatory

nerve endings (but not in astrocytes, see below) in hippocampal slices [18].

As observed previously [8, 9] we here show that in slices incubated with 3 mM K^+ the aspartate and glutamate antibodies produced a zonal labelling pattern corresponding to the nerve terminal field of the excitatory afferents in hippocampus (Fig. **1A**, **1B**). The aspartate and glutamate staining were mostly located in small dots (insets in Fig. **1A** and **B**) along weakly labelled dendritic structures. Electron microscopic immunogold cytochemistry [9] confirmed that the

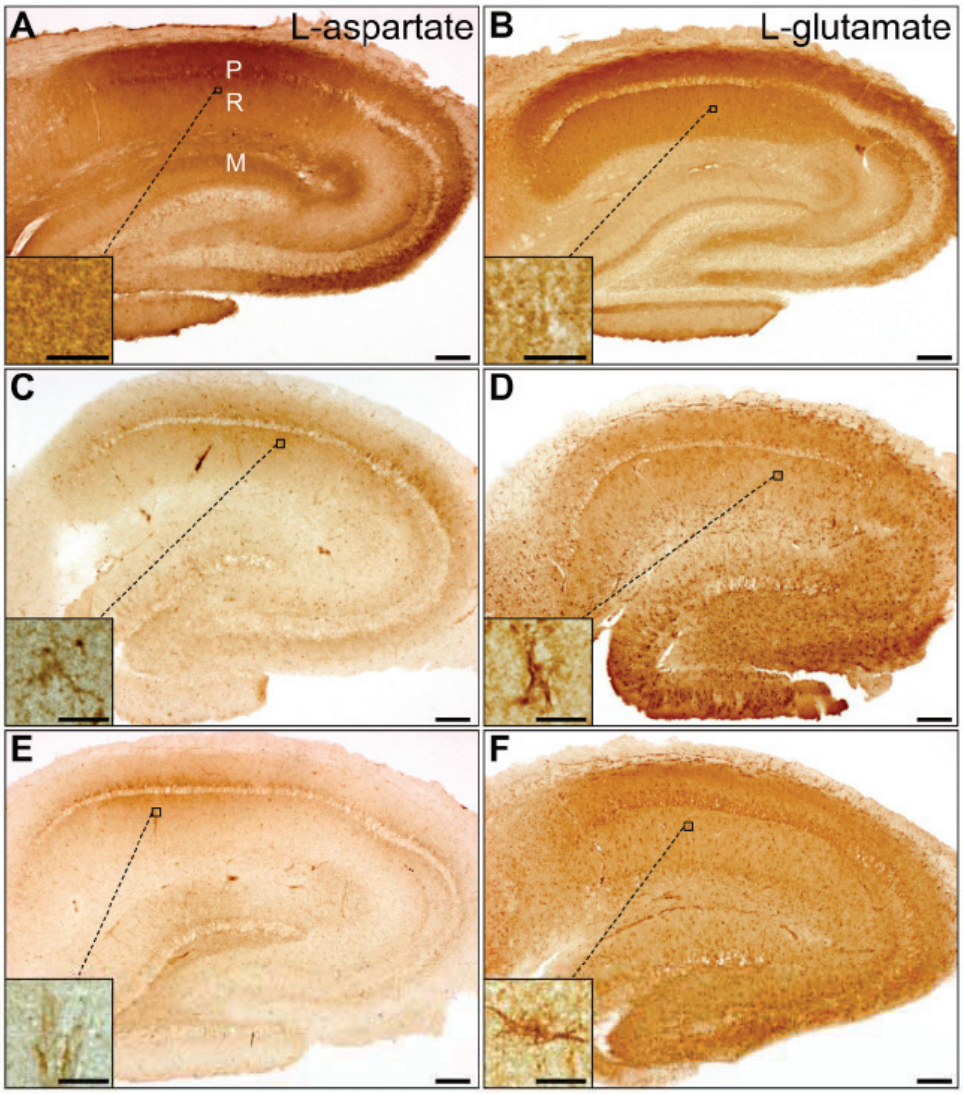

Fig. (1). The K^+-induced depletion of aspartate and glutamate from nerve ending-like dots was not substantially inhibited by PMB-TBOA. At physiological K^+-concentrations (3 mM) aspartate (**A**) and glutamate (**B**) immunoreactivities were located in dots (insets in **A** and **B**) corresponding to the excitatory nerve terminals in the hippocampus. At 55 mM K^+ the aspartate (**C**) and glutamate (**D**) staining mostly disappeared from the nerve terminal-like dots and accumulated in astroglial cells (insets in **C** and **D**), including in those around blood vessels (perivascular astroglia). At 55 mM K^+ in the presence of PMB-TBOA the aspartate (**E**) and glutamate (**F**) staining pattern was approximately unchanged, still showing that the most prominent staining was located in astroglial cells (insets in **E** and **F**). Similar aspartate and glutamate labelling patterns were observed in 6 slice experiments. Symbols: P, str. pyramidale; R, str. radiatum; M, dentate molecular layers. Bars: 200 µm, 20 µm (insets).

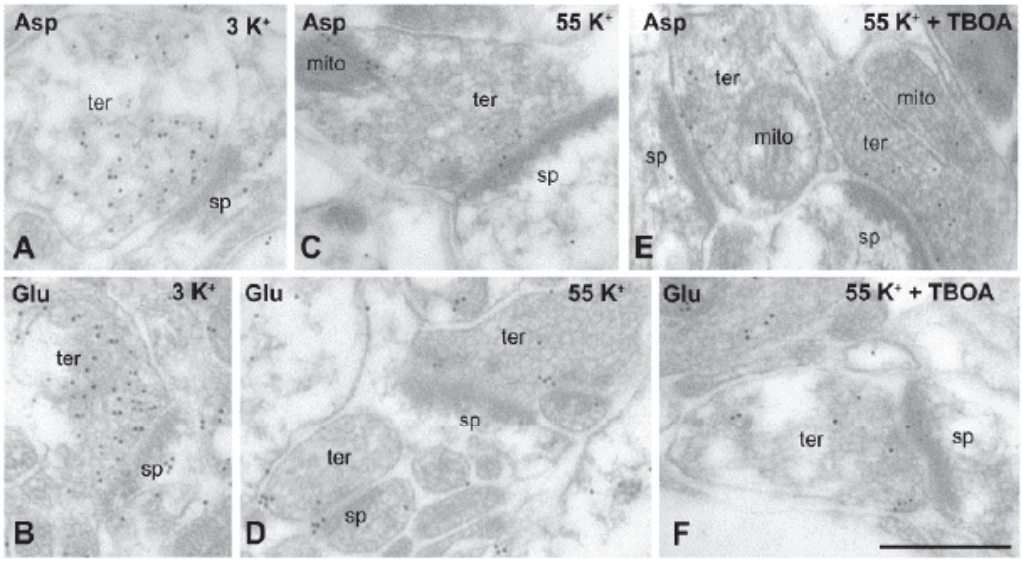

Fig. (2). Electron micrographs showing gold particles signalling aspartate (**A, C, E**) and glutamate (**B, D, F**) in excitatory synapses between nerve terminals (ter) and postsynaptic spines (sp) in hippocampal slices incubated at 3 mM K^+ (**A** and **B**), 55 mM K^+ (**C** and **D**) and 55 mM K^+ plus PMB-TBOA (**E** and **F**). Note that both aspartate and glutamate are depleted from the terminals upon stimulation with an elevated concentration of K^+ and that the treatment with PMB-TBOA does not block this depletion. Symbol: mito, mitochondria. Scale bar: 200 nm.

dotted staining represents labelling of nerve terminals making asymmetric synapses with dendritic spines (i.e. synapses with an "excitatory appearance") (Fig. **2A**, **2B**). Thus, we call the labelled dots nerve ending-like dots. When the slices were depolarised with a high K^+ concentration (50 mM) the aspartate and glutamate immunoreactivities were depleted from the nerve ending like dots. Instead, the staining appeared in astrocytes (Fig. **1C**, **1D**). Immunelectron microscopy of slices exposed to membrane depolarisation showed a robust reduction in the content of gold particles signalling aspartate and glutamate in excitatory nerve terminals (Fig. **2C**, **2D**). This was substantiated by quantitative immunogold analysis, which showed that K^+-induced depolarisation caused the nerve terminal densities of aspartate and glutamate immunogold particles to be significantly reduced (Fig. **3**). Taken together, this suggests that aspartate and glutamate is released from nerve terminals and taken up into astrocytes when the neurons are depolarised.

At 3 mM K^+ PMB-TBOA did not have any effect on the aspartate and glutamate labelling patterns (not shown). During depolarisation with 55 mM K^+, the slices which were treated with PMB-TBOA showed a redistribution from a nerve ending like staining pattern to an astrocyte like staining pattern (Fig. **1E**, **1F**), similar to that observed at 55 mM K^+ without PMB-TBOA (Fig. **1C** and **1D**). The uptake of aspartate and glutamate in astrocytes during K^+ stimulation in the presence of TBOA probably occurs through a sodium dicarboxylate transporter [18]. The immunogold labellings revealed that the densities of gold particles signalling aspartate and glutamate were somewhat higher in the depolarised terminals exposed to PMB-TBOA than in the terminals not exposed to PMB-TBOA, but lower than in the terminals from slices incubated at 3 mM K^+ (Figs. **2E**, **2F** and **3**). The immunogold quantifications (Fig. **3**) showed that about 30% and 36% of the L-aspartate and glutamate immunogold particles depleted from the terminals during depolarisation could be blocked by PMB-TBOA. This indicates that about 64-70 % of immunogold particles signalling L-aspartate and glutamate are depleted from the terminals due to exocytosis.

Localisation of D-Aspartate Immunogold Particles

As we found that the contribution from the EAATs for the total release of aspartate from nerve terminals was relatively minor, and several lines of evidence suggest that both L- and D-aspartate could be released by exocytosis (see Introduction), there should be a transporter capable of pumping L- and D-aspartate into synaptic vesicles (see Miyaji *et al.* 2008). To further investigate this uptake process we made use of a D-aspartate uptake assay, in which hippocampal slices were incubated with exogenous D-aspartate (50 μM), fixed with glutar- and formaldehyde and subjected to electron microscopic immunogold cytochemistry using specific antibodies against D-aspartate [16-18, 22]. As D-aspartate is very slowly metabolised [32], it is "trapped" in the cellular compartment into which it was taken up.

Slices not exposed to D-aspartate did not contain any significant D-aspartate immunogold signal (not shown, see [16-18, 22]). In contrast, slices exposed to 50 μM D-aspartate showed clear sign of D-aspartate uptake in excitatory nerve terminals (Fig. **4A** and **C**) (as well as in astrocytic processes (not shown, see [16]). A closer examination of the labelled terminals showed that D-aspartate gold particles were located over synaptic vesicles (insets in Fig. **4A** and Fig. **4B**). Interestingly, when quantifying the density of D-

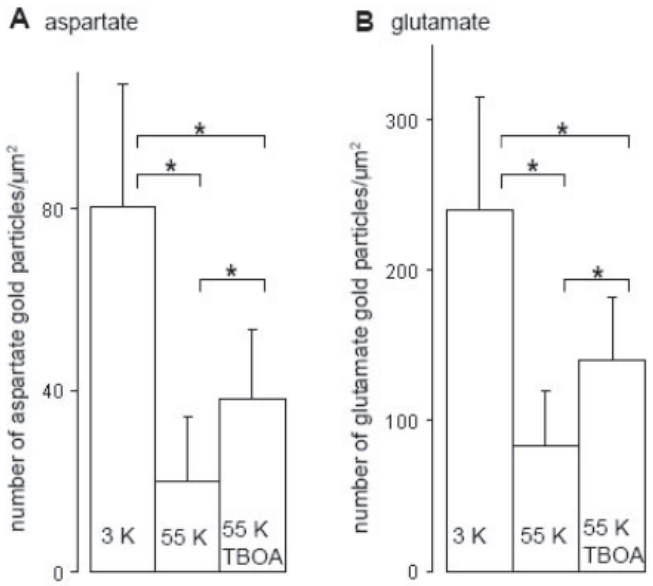

Fig. (3). Quantitative representation of the density of aspartate (**A**) and glutamate (**B**) immunogold particles in excitatory nerve terminals in hippocampal slices incubated with 3 mM K^+ (n=45 nerve terminals in **A** and **B**), 55 mM K^+ (n=40 terminals in **A** and **B**) and 55 mM K^+ in the presence of PMB-TBOA (n=42 nerve terminals in **A** and **B**). Asterisks denote that aspartate and glutamate values at 3 K^+ are significantly different from those at 55K^+ and 55K^+ + TBOA and that the values at 55K^+ are significantly different from those at 55K^+ + TBOA ($p<0.01$, Mann-Whitney-U test, two tails). The results are from one slice experiment. The same results were obtained in another experiment.

aspartate immunogold particles over synaptic vesicles and cytoplasmic matrix in the excitatory terminals (see Methods), we found that the gold particle density was much higher over the former than the latter compartment, giving a synaptic vesicle/cytoplasmic matrix ratio of about 9. From test sections with known concentrations of D-aspartate incubated with the D-aspartate antibodies in parallel with the hippocampal sections [22] we could estimate that the fixed D-aspartate concentration in the cytoplasmic matrix was about 2 mM, whereas the D-aspartate concentration in synaptic vesicles was about 18 mM.

DISCUSSION

Previously we have shown that the depletion of aspartate and glutamate from excitatory nerve terminals is sensitive to low Ca^{2+} conditions and tetanus toxin [9]. This result could be explained by the possibility that it is glutamate that is released from synaptic vesicles, whereas aspartate is released from the cytosol through EAAT mediated heteroexchange with synaptically released glutamate. In this way the aspartate release would seemingly appear as exocytotic. However, here we show that there is a substantial depletion of both aspartate and glutamate form excitatory nerve terminals during K^+ induced depolarisation, despite the presence of the EAAT blocker TBOA. This strongly suggests that release through the EAATs cannot account for the overall release of aspartate from the terminals, indicating that at a major part of the released aspartate during K^+ stimulation is derived from exocytosis of synaptic vesicles.

Our previous immunogold results from excitatory nerve terminals show that there is a residual depolarisation induced depletion of aspartate and glutamate, which is not blocked by conditions inhibiting exocytosis [9]. Thus, parts of the aspartate and glutamate release could be mediated by the EAATs. The non-exocytotic aspartate and glutamate depletion is about 30-40% of the total depletion during K^+ triggered depolarisation [9]. This fits with the present data showing that approximately 30-36% of aspartate and glutamate released from the excitatory terminals could be blocked by blocking the EAATs. These data suggest that a minor fraction of aspartate and glutamate released from the terminals escapes through the EAATs, whereas the largest fraction (about 70%) is released through exocytosis.

The present data, showing that the depletion of aspartate and glutamate from the nerve terminals is inhibited to a similar extent by blocking the EAATs, is compatible with the notion that the non-exocytotic release of the excitatory amino acids during K^+-induced depolarisation is caused by a reversal of the nerve terminal EAATs. This is in line with biochemical data, showing that the K^+-induced release of newly taken up D-aspartate from hippocampal nerve endings occurs in part by exocytosis (about 65%) and in part by transporter reversal (about 35%) [12].

Our finding that the ratio of D-aspartate labelling between synaptic vesicles and cytoplasmic matrix was about 9 supports the idea that aspartate can be taken up into synaptic vesicles before exocytotic release. This uptake is mediated by other transporters than the vesicular glutamate transport-

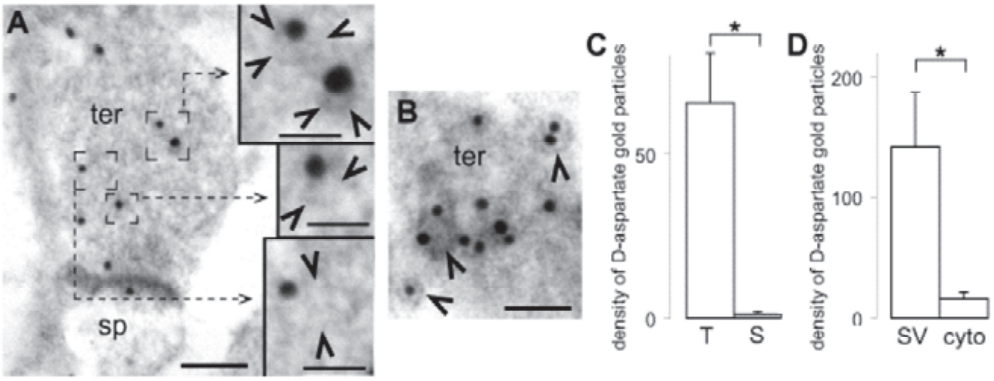

Fig. (4). D-aspartate is taken up into synaptic vesicles in excitatory nerve terminals. The electron micrographs (**A, B**) show gold particles representing D-aspartate in nerve terminals (ter) making asymmetric synapses with postsynaptic spines (sp) in hippocampal slices exposed to exogenous D-aspartate at resting conditions (3 mM K^+). Note the clear association between the D-aspartate immunogold particles and synaptic vesicles (arrowheads in the insets in **A** and in **B**). Scale bars: 100 μm in **A**, 50 μm in insets and in **B**. **C** shows the density of D-aspartate gold particles (average number of gold particles/μm^2 ± SD) in 20 excitatory synapses between nerve terminals (T) and spines (S). **D** shows the density of D-aspartate gold particles in synaptic vesicles (SV) and cytoplasmic matrix (cyto) in the 20 nerve terminals represented in **C**. Asterisks denote that the D-aspartate value in T is significantly different from that in S and that the value in SV is significantly different from that in cyto ($p<0.01$, Mann-Whitney-U test, two tails). The quantifications are from one slice experiment. Similar results were obtained in two other experiments.

ers (VGLUTs), which neither transport L-aspartate nor D-aspartate [33-39]. Even though Miyaji *et al*. [15] did not give any information about vesicular D-aspartate uptake, the present data are in good agreement with their identification of a vesicular excitatory amino acid transporter that carries both aspartate and the aspartate analogue threo-hydroxy aspartate [5] into hippocampal synaptic vesicles [15]. Our results also support those of Fleck and co-workers [14], who showed that radiolabelled L- and D-aspartate could be taken up in synaptic vesicles, which were immunoisolated with synaptophysin after amino acid preincubation of intact synaptosomes. However, it should be noted that especially the L-aspartate uptake data from this study should be interpreted with caution. During the preincubation of the synaptosomes some of the radiolabel on L-aspartate could have been transferred to L-glutamate through metabolism in the Krebs' cycle. However, as D-aspartate is very slowly metabolised in the brain [32], the possibility that such a mechanism underlies the result of the D-aspartate uptake experiments of [14] is not very likely. Thus, the present results and those of Fleck and co-workers [14] strongly suggest that also D-aspartate is transported into synaptic vesicles, probably by the same transporter as L-aspartate. This is in line with the large body of evidence showing exocytosis of both L-aspartate [1-4, 6-10, 14] and D-aspartate [11, 12, 40-45].

We found that the cytoplasmic concentration of D-aspartate (about 2 mM) after uptake into nerve terminals was in the range of the apparent Km values for aspartate uptake into synaptic vesicles reported both by Miyaji *et al*. [15] and Fleck *et al*. [14]. The substrate specificity of VEAT indicates that it carries both glutamate and aspartate. This may explain the previous immunogold findings of glutamate and aspartate in the same nerve terminals [9, 27]. There are so far no detailed electron microscopic localisation studies of VEAT in the brain, only previous light microscopic results showing that the sialin protein (see Introduction) is present at quite high concentrations throughout the forebrain, including in the hippocampus [46, 47]. Whether vesicles containing VEAT is located in the same terminals as those containing VGLUT is one important question that must await further studies.

The conclusion from previous uptake studies using whole brain isolated synaptic vesicles is that the vesicles do not transport aspartate [48-52]. However, most of these studies did not analyse direct uptake of radiolabelled aspartate, but showed that neither L- nor D-aspartate could inhibit uptake of L-glutamate into synaptic vesicles. This indicates that L- and D-aspartate are not transported by the same transporter proteins as glutamate, but does not exclude the possibility that aspartate is transported by another vesicular transporter, such as VEAT [15]. It should, however, be noted that the initial study of Naito and Ueda [48] showed that whole brain synaptic vesicles could indeed accumulate L-aspartate, although the uptake was much lower (7.5%) than that of L-glutamate. In the studies of Miyaji *et al*. [15] threo-hydroxy aspartate could not significantly inhibit glutamate uptake into whole brain synaptic vesicles (only into hippocampal vesicles), indicating that VEAT is much less abundant that the VGLUTs.

CONCLUSION

We here show that excitatory synaptic vesicles in the hippocampus can accumulate D-aspartate and release L-aspartate by exocytosis, further corroborating the evidence for a vesicular aspartate transporter [15] in these vesicles.

ACKNOWLEDGEMENT

The work was supported by grants from the Medical Faculty, University of Oslo, the Norwegian Research Coun-

cil, the South-Eastern Norway Regional Health Authority and from Rikshospitalet University Hospital.

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Received: September 01, 2008 Revised: October 08, 2008 Accepted: October 15, 2008

II

III

Valproate causes reduction of the excitatory amino acid aspartate in nerve terminals

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Key words:
aspartate
valproate
epilepsy
neurotransmission
nerve terminal
excitotoxicity

ABSTRACT

Valproate is well established in the treatment of epilepsy and psychiatric disorders, yet the main mechanism of action remains to be determined. Here we show that valproate may reduce neurotransmission of the excitatory amino acid, aspartate. By electron microscopic immunogold cytochemistry we demonstrate a 68% reduction in the level of aspartate in excitatory nerve terminals at 30 minutes after an acute dose of valproate. The level of glutamate in excitatory nerve terminals, and the aspartate and glutamate levels in stem dendrites were not significantly changed by valproate treatment. In inhibitory terminals, valproate caused a 65% decrease in the aspartate level, whereas the GABA level was unchanged. The present study show that valproate alters the nerve terminal content of the excitatory neurotransmitter aspartate. This suggests that the releasable pool of aspartate is regulated separately from the somato-dendritic pool in response to valproate treatment, and points to a new mechanism of action for valproate.

Submitted

Introduction

Valproate is one of the most widely used drugs in neurology and psychiatry, and has been used in the treatment of epilepsy for more than 30 years. Yet its main mechanism of action is still unclear. Valproate has a broad spectrum of effects against both generalised and partial seizures, as well as against bipolar disorders [38]. Several mechanisms have been proposed to explain the clinical action of valproate, including inhibition of voltage-gated sodium channels [31;40], inhibition of neuronal energy metabolism [21] and effects on neurotransmitter amino acids [32;33]. At therapeutically relevant concentrations, it is unclear if direct inhibition of voltage-gated sodium channels contributes to the clinical effect of valproate (for review, see [20;27]). Instead, increased GABAergic transmission is believed to be highly important both for the acute and long-term effects of valproate. It is well known that GABA accumulates in the brain after valproate treatment [1;11;26;29;30]. The increase in GABA is thought to occur selectively in GABAergic terminals [26].

Valproate also causes the brain levels of the excitatory transmitter aspartate to decrease [22;39]. The effect of valproate on the distribution of aspartate between different subcellular neuronal compartments, however, remains unknown. If valproate predominantly reduces aspartate levels in nerve terminals, the transmitter releasing compartment, this would suggest that valproate leads to reduced excitatory synaptic activity, in turn pointing to an additional mechanism for the clinical action of valproate.

Materials and Methods

Animal handling was in strict accordance with local and national ethical guidelines. Male Wistar rats (n= 3 in each group) were given an intraperitoneal (i.p.) injection of sodium valproate (400 mg/kg) or saline (0.9%). At 30 minutes after the injection, the rats were anaesthetised with pentobarbital (100 mg/kg, i.p.) and transcardially perfused with a mixture of 2.5% glutaraldehyde and 1% paraformaldehyde in phosphate buffer (pH 7.4). The brains were gently removed, and the hippocampal CA3 region was dissected out and embedded in Lowicryl HM20 as previously described [2]. After embedding, ultrathin sections (80-100 nm) were cut and labelled with the 435 L-aspartate (1:300), 607 L-glutamate (1:3000) or 990 GABA (1:300) antisera. These antisera have been well characterised [4;12-14;18;19]. To avoid possible cross-reactivities, the glutamate, aspartate and GABA antisera were used with the addition of soluble complexes (0.2 mM) of glutaraldehyde/formaldehyde-treated L-asp ate plus glutamine, and L-asparagine, L-glutamate plus GABA, and L-glutamate, L-aspartate plus β-alanine, respectively. As a specificity test, ultrathin test sections containing various amino acids conjugated to brain protein by form- and glutaraldehyde [36] were labelled along with the tissue sections. These test systems showed that the primary antibodies only labelled the conjugate containing the amino acid against which the antibodies were raised (not shown). The primary antibodies were visualised with colloidal gold conjugated goat anti-rabbit IgG (British Biocell International; Cardiff, UK). The sections were studied in a Tecnai 12 electron microscope. To visualise aspartate and glutamate/GABA in the same nerve terminals, we performed double labelling experiments, in which the ultrathin sections were first treated with the aspartate antibodies and then with the glutamate or GABA antibodies. Between the first and the second step, the sections were subjected to formaldehyde vapour (80°C, 1 hour) to prevent interference between the sequential incubations [37]. Secondary antibodies coupled to 10 and 15 nm gold particles were used in the first and second step, respectively. Electron micrographs were randomly taken from the CA3 stratum radiatum, granual- or pyramidal cell layer. The terminals to be included in the study were chosen at low magnification, where the 10 nm aspartate gold particles were

were not visible, and all quantitative analysis were performed by a blinded observer (CM). The densities (number of gold particles/ μm^2) of aspartate and glutamate gold particles in excitatory nerve terminals, dendritic spines and stem dendrites and the densities of aspartate and GABA gold particles in inhibitory terminals, were calculated as described [24]. In excitatory nerve terminals gold particle densities were separately determined in the cytosol and mitochondria, while in the other tissue profiles gold particle densities were recorded in the cytosol. Background labelling over empty resin in each section was subtracted.

Excitatory terminals were defined as those making asymmetric synapses with dendritic spines and containing glutamate immunogold particles. Inhibitory terminals were defined as those making symmetric synapses with stem dendrites or granule cell bodies and containing GABA immunogold particles. The results were statistically evaluated by a non-parametric Mann Whitney-U test, two tails (SPSS). Data are given for one labelling experiment, in which sections from 3 controls and 3 valproate hippocampi were analysed, but an additional labelling experiment for aspartate/glutamate and two experiments for aspartate/GABA produced similar results.

Results

To determine if valproate regulates the nerve terminal pool of aspartate differently from the dendritic pool, we used vaproate treated rats and controls to quantify aspartate immunreactivities in excitatory terminals, inhibitory terminals, dendritic spines and stem dendrites in the stratum radiatum of CA3 hippocampus. As observed previously in the CA1 stratum radiatum [12;18], we found that aspartate immunogold particles were located together with glutamate immunogold particles in excitatory terminals (Fig. 1) and that the density of gold particles signalling aspartate and glutamate was higher in excitatory terminals than in stem dendrites (Fig. 1).

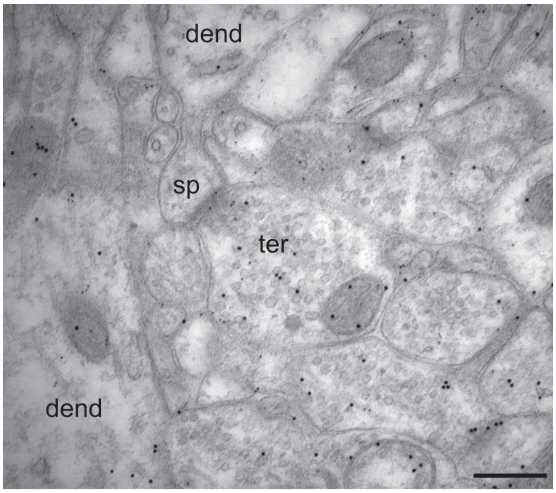

Figure 1: *Low power electron micrograph showing aspartate (small gold particles) and glutamate (large gold particles) labelling of nerve terminals (ter), dendritic spines (sp) and stem dendrites (dend) in the CA1 stratum radiatum. Scale bar=300 nm*

In response to valproate, aspartate levels were profoundly decreased in excitatory terminals, but not in stem dendrites (Fig. 2A vs. 2C and 2B vs. 2D). Quantitative analyses showed that, in excitatory terminals, valproate reduced the density of gold particles representing aspartate by 68% (Fig. 2E), while the glutamate level was not significantly reduced (Fig. 2E). The area of nerve terminals was not affected by valproate treatment (0.23 ± 0.04 μm^2 in the control group vs. 0.27 ± 0.01 µm2 in the valproate treated group; average area ± SEM, n=3 animals; p>0.05 (Mann Whitney-U test, two tails)). In stem dendrites, neither aspartate nor glutamate levels were significantly changed after valproate treatment (Fig. 2F). However, gold particle densities (average number of gold particles ± SEM, n=3 animals) in dendritic spines paralleled the changes detected in the opposing nerve terminals: Aspartate levels decreased from 29.5 ± 4.0 gold particles/μm^2 in the control group to 7.8 ± 2.2 gold particles/μm^2 in the valproate treated group ($p<0.05$, Mann Whitney-U test, two tails), while the glutamate levels were not altered by the treatment (32.4 ± 3.5 gold particles/ μm^2 in the controls vs. 27.4 ± 10.0 gold particles/μm^2 in the valproate treated group; p>0.05, Mann Whitney-U test, two tails). In excitatory nerve terminals the mitochondrial density of aspartate gold particles was 42% lower in valproate treated rats than in controls, but this differences did not reach statistical significance (39.0 ± 7.1 vs. 22.6 ± 12.2 gold particles/μm^2; average number of gold particles ± SEM, n=3 animals; p>0.05, Mann Whitney-U test, two tails). The densities of glutamate gold particles, were largely unchanged (79.7 ± 6.4 vs.62.1 ± 20.4 gold particles/μm^2; average number of gold particles ± SEM, n=3 animals; p>0.05, Mann Whitney-U test, two tails). As reported before [14] we found that inhibitory nerve terminals contained strong aspartate labelling (Fig. 3A). Like in excitatory nerve terminals, valproate reduced the labelling for aspartate in inhibitory terminals (Fig. 3B). Immunogold quantification showed that in these terminals the density of aspartate gold particleswas reduced by 65% in response to valproate treatment, while the GABA level was unchanged (p>0.05, Mann Whitney-U test, two tails) (Fig. 3C). The level of aspartate in inhibitory terminals was approximately twice as high as in excitatory terminals (Fig. 3C vs. 2E).

Discussion

The present study demonstrates that valproate causes a selective decrease in the nerve terminal pool of aspartate compared to the pool in stem dendrites, strongly suggesting that valproate affects the release of aspartate. Moreover, in excitatory nerve terminals, only aspartate and not glutamate, was significantly reduced. The decreased nerve terminal levels of aspartate described in this study are likely to cause diminished aspartergic neurotransmission, as the degree of vesicular filling is dependent on the cytosolic concentration of neurotransmitter [42]. This is in line with biochemical studies showing that valproate selectively inhibits the release of aspartate in preference to glutamate [3;6]. Further supporting the notion that valproate decreases release of aspartate from terminals, is the finding that the aspartate and glutamate content in dendritic spines paralleled the levels in nerve terminals. Since spines do not contain mitochondria and therefore are unable to synthesise their own amino acids, we would expect that the aspartate levels in spines should be similar to the levels in stem dendrites. The fact that this was not the case could be explained by the ability of spines to take up aspartate from the synaptic cleft through EAAT3, which is a glutamate/aspartate transporter concentrated at the postsynaptic edge and perisynaptic sites in hippocampal spines [16]. This suggests that the amino acid levels in the spines to a large degree reflect uptake of amino acids released from the nerve terminals. Thus, at the synapse, reduced release of aspartate would lead to reduced aspartate content in spines.

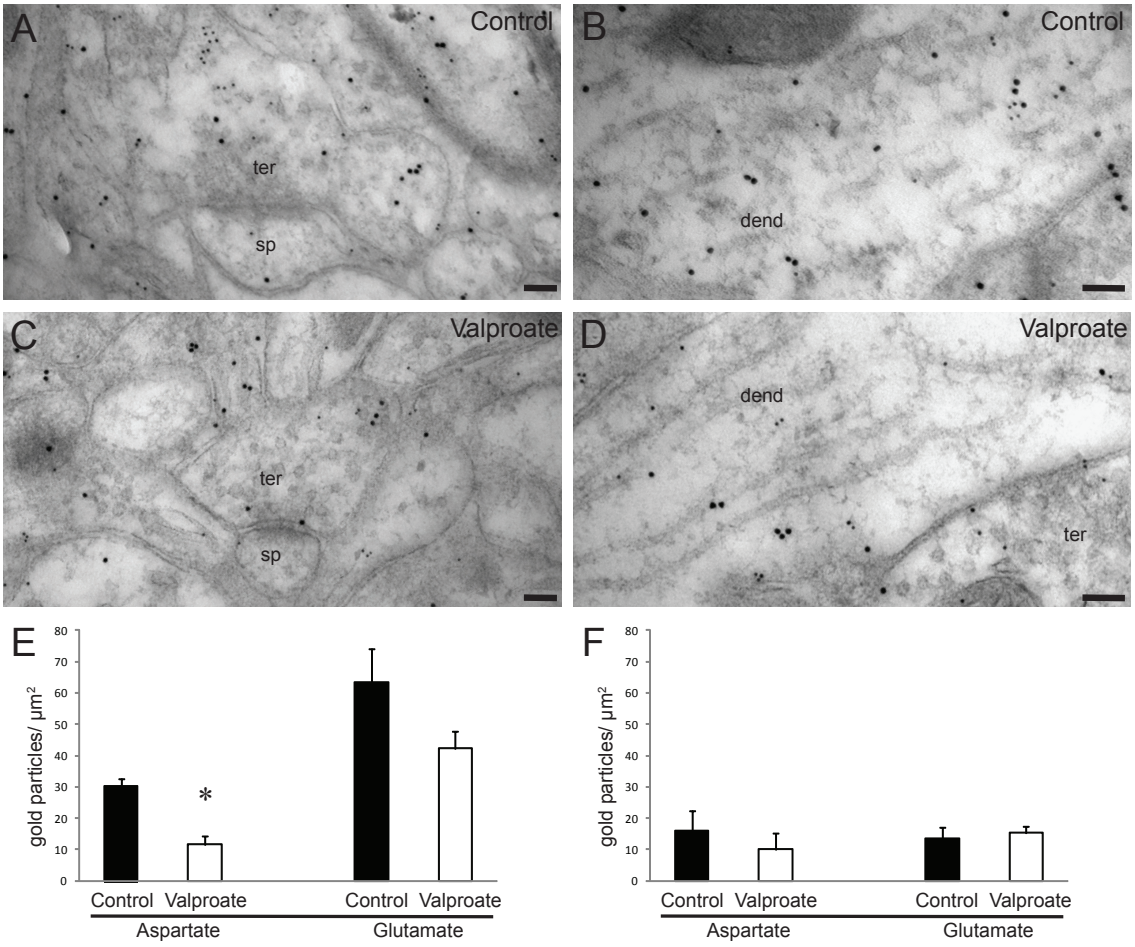

Figure 2: *Valproate reduces the level of the excitatory amino acid aspartate in excitatory nerve terminals. Electron micrographs showing aspartate (small gold particles) and glutamate (large gold particles) labelling of nerve terminals (ter) making asymmetric synapses with dendritic spines (sp) (A and C) and of stem dendrites (dend) (B and D) in the stratum radiatum of CA3 hippocampus from saline treated (controls) (A and B) and valproate treated rats (C and D). Scale bars=100 nm. Quantitative assessment of aspartate and glutamate in excitatory terminals (E) and stem dendrites (F). The values indicated by the bar charts are mean number of gold particles/µm² ± SEM of n=3 rats (26-32 excitatory terminals; 9-15 stem dendrites per animal). *Aspartate values in valproate treated terminals (9.5 ± 2.9) were significantly lower than the values in control terminals (30.1 ± 2.5; p<0.05, Mann Whitney-U test, two tails (SPSS)). The glutamate values in control terminals (63.3±10.9) and valproate treated terminals (47.2 ± 8.9), as well as aspartate and glutamate values in stem dendrites (16.0 ± 6.3 and 13.5 ± 3.7 in controls vs. 10.0±5.4 and 15.3±2.1 in valproate treated animals) were not significantly altered by valproate treatment (p>0.05, Mann Whitney-U test, two tails (SPSS)).*

Our finding that valproate caused a reduction in aspartate is in agreement with previous biochemical data from the hippocampus [5;28]. Using valproate at doses of 200-400 mg/kg, the two latter studies found that valproate reduced hippocampal aspartate levels by about 20-30%, while Johannessen et al. [20] and Kukino and Deguchi [22] found about 45% drop in aspartate levels in the whole brain after valproate treatment (400 mg/kg). The reason why we observed a stronger reduction in aspartate than reported in the biochemical studies is that aspartate is only reduced in synaptic structures; the biochemical studies measured the total content of aspartate, including the somato-dendritic content, which did not show any alteration after valproate treatment.

The decrease in aspartate levels were accompanied by insignificant changes in glutamate and GABA, suggesting that valproate selectively regulates the release of aspartate over that of glutamate and GABA. These are important findings as they substantiate previous results showing release of aspartate at excitatory [12;18] and inhibitory [14] synapses and support the notion that the release of aspartate and other neuroactive amino acids could be regulated by different mechanisms [35]. Aspartate released into the extracellular space selectively activates the NMDA type of glutamate receptors [8]. These receptors are located at most excitatory synapses in the hippocampus [41], but also at inhibitory synapses [14]. The attenuated release of aspartate caused by valproate could therefore act to reduce NMDA receptor signalling and account for part of the antiepileptic or mood stabilizing effect of valproate. Interestingly, by electrophysiology it has been found that valproate decreases NMDA receptor responses via a pre-synaptic mechanism [7;10]. Further supporting the idea that valproate targets aspartate release, is that valproate

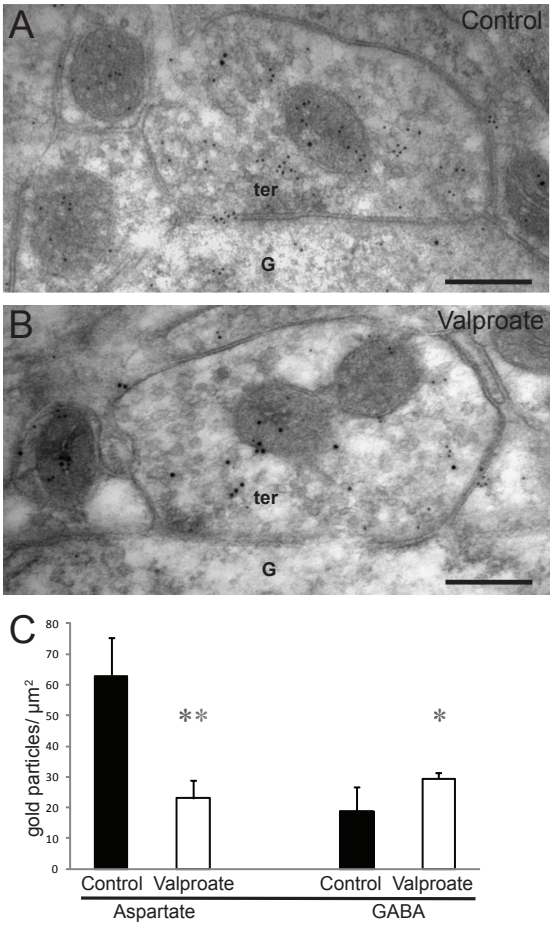

Figure 3: *The level of the excitatory amino acid aspartate is reduced in inhibitory nerve terminals after valproate treatment. Electron micrographs showing aspartate (small gold particles) and GABA (large gold particles) labelling in inhibitory nerve terminals (ter) on granule cell bodies (G) in the CA3 hippocampus from a saline treated (control) (A) and a valproate treated rat (B). Scale bars=100nm. Quantitative assessment of aspartate and GABA in inhibitory terminals (C). The values indicated by the bar charts are mean number of gold particles/µm² ± SEM in n=3 rats (20-25 terminals from each animal were analysed). *Aspartate values in valproate treated terminals (24.5 ± 5.2) were significantly lower than the values in control terminals (63.7 ± 12.0; p<0.05, Mann Whitney-U test, two tails (SPSS)), while GABA values in control terminals (22.0 ± 6.40) and valproate treated terminals (28.0 ± 2.26) were not significantly different (p>0.05, Mann Whitney-U test, two tails (SPSS)).*

inhibits the increased aspartate release and seizures known to occur in epileptic El-mice [17]. Also, in pentylenetetrazol kindled rats, aspartate release during seizures is reduced by valproate [25].

What is the mechanism for the valproate induced decrease in nerve terminal aspartate? Valproate inhibits the intramitochondrial enzyme α-ketoglutarate dehydrogenase [20], which leads to reduced concentration of oxaloacetate and thereby of aspartate, which is formed from oxaloacetate via the aspartate aminotransferase reaction. The selective effect in nerve terminals might be due to a higher turnover of transmitter amino acids in nerve terminals than in somatodendritic compartments [9].

It should also be noted that the labelling density of aspartate in inhibitory terminals was approximately twice of that in excitatory terminals. This is in agreement with several studies showing higher aspartate levels in GABAergic neurons than in glutamatergic neurons [13;13;15;15], and probably have a metabolic explanation: One of the rate limiting enzymes of the oxidative metabolism is α-ketoglutarate dehydrogenase [23;34]. In GABAergic neurons, the GABA-shunt can circumvent the step catalyzed by α-ketoglutarate, facilitating the flux through to oxaloacetate. The conversion of oxaloacetate to citrate, however, is dependent of acetyl-CoA formed by pyruvate dehydrogenase, whose capacity is as limited as that of α-ketoglutarate dehydrogenase [34]. Thus oxaloacetate, and subsequently aspartate, accumulates in these neurons.

In conclusion, we show that valproate leads to a decrease in the nerve terminal content of the excitatory amino acid aspartate in hippocampus, suggesting that valproate acts through reducing NMDA receptor mediated excitatory signalling in the brain.

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