Olav Bjerkehagen Smeland

Brain Metabolism in Mice Supplemented with Acetyl-l-carnitine and Two Mouse Models of Temporal Lobe Epilepsy

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
Faculty of Medicine
Department of Neuroscience

NTNU – Trondheim
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Metabolismen i hjernen til mus behandlet med acetyl-l-carnitine og to musemodeller for temporallappsepilepsi

Sykdommer i hjernen, som epilepsi og depresjon, er vanlige. Ikke bare påvirker disse sykdommene livene til den enkelte pasient og de pårørende, men de utgjør også store utfordringer for samfunnet. Felles for de fleste hjernesykdommene er at vi i liten grad kjenner til de underliggende mekanismene som ligger bak dem. Derfor er det også vanskelig å utvikle ny og effektiv behandling. Vi trenger med andre ord mer forskning for å forstå bedre hvordan hjernen fungerer og hvordan sykdommene arter seg.

I min doktorgrad har jeg arbeidet med to musemodeller med epilepsi og et næringsstoff, acetyl-l-carnitine. Målet med avhandlingen har vært å undersøke endringer i stoffskiftet i hjernen til mus med epilepsi og hvordan acetyl-l-carnitine påvirker stoffskiftet i hjernen både til friske mus og mus med epilepsi. Ved hjelp av blant annet avansert magnetisk resonans (MR) teknologi har jeg målt nivåene av ulike stoffer i hjernen til mus.

Resultatene av arbeidet mitt viser at de epileptiske musene hadde reduserte nivåer av det vanligste signalstoffet i hjernen, glutamat, i området hippokampusformasjonen. Videre tyder flere funn på at funksjonen til mitokondriene i den epileptiske hjernen er redusert. Acetyl-l-carnitine forebygget ikke kramper hos epileptiske mus, men motvirket visse spesifikke endringer i stoffskiftet i hjernen til disse musene. I friske mus økte acetyl-l-carnitine energilagrene i hjernen samt nivåene av to viktige signalstoffer i hjernen, noradrenalin og serotonin, som begge spiller sentrale roller i behandlingen av depresjon.

Min forskning har gitt mer innsikt i endringer i hjernen ved epilepsi som kan gi grunnlag for bedre diagnostikk og behandling av sykdommen. I tillegg gir resultatene grunnlag for videre utprøving av acetyl-l-carnitine i behandlingen av ulike hjernesykdommer.

Navn kandidat: Olav Bjerkehagen Smeland
Institutt: Institutt for Nevromedisin
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He remembered that during his epileptic fits, or rather immediately preceding them, he had always experienced a moment or two when his whole heart, and mind, and body seemed to wake up to vigor and light; when he became filled with joy and hope, and all his anxieties seemed to be swept away for ever; these moments were but presentiments, as it were, of the one final second (it was never more than a second) in which the fit came upon him. That second, of course, was inexpressible… That there was, indeed, beauty and harmony in those abnormal moments, that they really contained the highest synthesis of life, he could not doubt, nor even admit the possibility of doubt… These instants were characterized—to define it in a word—by an intense quickening of the sense of personality. Since, in the last conscious moment preceding the attack, he could say to himself, with full understanding of his words: "I would give my whole life for this one instant"…

Excerpt from the novel "The Idiot" by Fyodor Mikhailovich Dostoyevsky, written in 1869, in which the experience of an epileptic seizure is described.

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There is a saying that the apple does not fall far from the tree. I am probably not an exception. Mom and dad, it would be a lie to say that I have not benefitted from your experiences and knowledge when encountering challenges in my research. I deeply thank you for your continuous support. Frode and Sunniva: high-five! Your brother has written a PhD thesis. I also want to thank my great-aunt Synnøve for caring for me and serving me countless delicious dinners during my stay here in Trondheim.

Finally a big thanks to all my friends who have encouraged me during my research and diverted my mind to other things in life that, admittedly, at times have seemed more exciting than research.

Abbreviations

α-ABA	L-2-aminobutyric acid
α-KG	α-ketoglutarate
AAT	aspartate aminotransferase
ADP	adenosine diphosphate
ALCAR	acetyl-l-carnitine
AMP	adenosine monophosphate
AQP4	aquaporin-4
ATP	adenosine-5'-triphosphate
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase
GAD	glutamate decarboxylase
GC-MS	gas chromatography–mass spectrometry
GDH	glutamate dehydrogenase
GFAP	glial fibrillary acidic protein
Gln	glutamine
Glu	glutamate
GS	glutamine synthetase
HF	hippocampal formation
HPLC	high-pressure liquid chromatography
MTLE	mesial temporal lobe epilepsy
NAA	*N*-acetyl aspartate
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NAD(P)H	reduced form of nicotinamide adenine dinucleotide and NADPH
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser enhancement
PAG	phosphate activated glutaminase
PC	pyruvate carboxylase
PDH	pyruvate dehydrogenase
PPP	pentose phosphate pathway
PTZ	pentylenetetrazole
SE	status epilepticus
SSADH	succinate semialdehyde dehydrogenase
TCA cycle	tricarboxylic acid cycle
TLE	temporal lobe epilepsy
TSP	2,2,3.3-d(4)-3-(trimethylsilyl)propionic acid sodium salt

Sammendrag

Epilepsi er en av de vanligste nevrologiske sykdommene i verden og temporallappsepilepsi (TLE) er sannsynligvis den hyppigste formen for epilepsi. Behandlingen av TLE er i dag ikke god nok og opp til 60% av personer med TLE blir resistente mot gjeldende epilepsimedisiner. Selv om tilstanden ofte kan kureres med kirurgi, har sosiale og psykiske ringvirkninger av tilstanden gjerne manifestert seg lenge før kirurgi gjennomføres. Mekanismene bak TLE er ennå ikke klarlagte, men mye tyder på at forstyrret metabolisme, særlig i hjernestrukturen hippocampus, bidrar til patogenesen. Ved å belyse disse metabolske endringene kan vi oppdage nye mål for behandling av TLE samt forbedre diagnostikken. I Artikkel 2 og 3 undersøkte vi metabolismen i hjernen til to modeller for TLE i mus, henholdsvis pentylenetetrazole (PTZ) kindling modellen og pilocarpine status epilepticus modellen. I tillegg evaluerte vi de antiepileptiske virkningene til acetyl-l-carnitine (ALCAR) på anfall og metabolisme i PTZ kindling modellen.

ALCAR er et kosttilskudd som er tilgjengelig i helsekostbutikker i utlandet men ikke i Norge. ALCAR finnes naturlig i kroppen og er involvert i frakten av acetyl-enheter over den mitokondrielle membranen. I mer enn tjue år har ALCAR blitt prøvd ut som terapeutisk virkemiddel ved ulike sykdommer i hjernen. Blant annet har behandling med ALCAR til pasienter med depressive tilstander gitt gode resultater. Administrering av én enkelt dose ALCAR til rotter gir økt energitilgjengelighet i hjernen og beskyttelse mot nevrotoksiske insulter som iskemi og oksidativt stress. Det har derimot vært en mangel på studier som viser langtidsvirkningene av ALCAR behandling på metabolismen i hjernen. I Artikkel 1 undersøkte vi virkningen av ALCAR gitt til mus i 25 dager på energi og nevrotransmitter homeostasen i hjernen.

I alle de tre studiene i denne avhandlingen skaffet vi detaljert oversikt over metabolske endringer i hjernebarken og hippokampusformasjonen (HF) til mus ved hjelp av ^{1}H nukleær magnetisk resonans (NMR) spektroskopi og væskekromatografi. For å studere metabolismen av glukose injiserte vi mus med ^{13}C merket glukose og undersøkte hjerneekstrakt med ^{1}H og ^{13}C NMR spektroskopi samt gass kromatografi-masse spektrometri.

I Artikkel 1 fant vi at tilskudd med ALCAR økte nivåene av høy-energi fosfater i hjernebarken til mus, noe som tyder på økt energitilgjengelighet. I begge hjerneområder senket ALCAR forbruket av glukose vist ved økte nivåer av glukose og reduserte nivåer av [3-^{13}C]laktat. Videre fant vi at ALCAR økte konsentrasjonene av monoaminene noradrenalin i HF og serotonin i hjernebarken. Disse funnene er særlig interessante i forhold til en potensiell antidepressiv virkning av ALCAR.

I artikkel 2 og 3 demonstrerte vi metabolske endringer i epileptiske mus som likner på funnene som er rapportert hos mennesker med TLE, deriblant lavere konsentrasjon av glutamat og mitokondriell dysfunksjon i HF. Vi viste også at omsetningen av viktige metabolitter som tilhører eller er derivert fra sitronsyresyklus var nedsatt i begge hjerneområder i epileptiske mus, forenlig med en dysfunksjonell sitronsyresyklus. Tilskudd med ALCAR påvirket ikke anfallsutviklingen i PTZ injiserte mus, men motvirket noen metabolske endringer.

Alt i alt har studiene skaffet ny informasjon om de metabolske endringene i hjernen til epileptiske mus og mus behandlet med ALCAR i 25 dager. Funnene vil kunne være nyttige for å forbedre diagnostikken og behandlingen av TLE og evaluere den potensielle rollen til ALCAR i behandlingen av ulike hjernesykdommer.

Summary

Epilepsy is one of the most prevalent neurological diseases in the world and temporal lobe epilepsy (TLE) is likely the most common form of epilepsy. The treatment strategies for TLE are not satisfactory and as many as 60% of patients with TLE become resistant to currently available antiepileptic drugs. Although the condition can be effectively treated by surgical resection, social and psychological disabilities of the disorder often manifest themselves long before referral to surgery. The mechanisms underlying TLE still remain unclear, although accumulating evidence points to metabolic dysfunction especially in the hippocampus. By elucidating these metabolic alterations, we may reveal new targets for antiepileptic treatment and improve diagnostic criteria. In Papers 2 and 3, we investigated brain metabolism in two mouse models of TLE, namely the pentylenetetrazole (PTZ) kindling model and the pilocarpine status epilepticus model, respectively. Moreover, the antiepileptic efficacy of acetyl-l-carnitine (ALCAR) on seizures and metabolism was evaluated in the PTZ kindling model.

ALCAR is a dietary supplement readily available in health food stores abroad but not in Norway. It is an endogenous compound involved in the transport of acetyl-moieties across the mitochondrial membrane. For more than two decades, ALCAR has been evaluated as a therapeutic supplement in various brain disorders, and has shown beneficial effects in small trials with patients with depressive disorders. In rats, single administration of ALCAR increases brain energy availability and protects against various neurotoxic insults such as ischemia and oxidative stress. However, there has been a lack of studies on the effect of chronic ALCAR administration on brain metabolism. In Paper 1, we investigated brain glucose, energy and neurotransmitter homeostasis in mice supplemented with ALCAR for 25 days.

In all three studies constituting this thesis, we obtained detailed maps of the metabolic content in the cerebral cortex and hippocampal formation (HF) of mice using ^{1}H nuclear magnetic resonance (NMR) spectroscopy and high-pressure liquid chromatography. To determine glucose metabolism, we injected mice with ^{13}C labeled glucose and evaluated brain extracts using ^{1}H and ^{13}C NMR spectroscopy and gas chromatography–mass spectrometry.

In Paper 1, we found that ALCAR supplementation increased the amounts of high-energy phosphates in the cortex of mice, indicating increased energy availability. In both brain regions, ALCAR decreased glucose consumption evidenced by increased amounts of glucose and decreased amounts of [3-^{13}C]lactate. Furthermore, ALCAR increased the concentrations of monoamines noradrenaline in the HF and serotonin in cortex. These findings are particularly interesting in relation to a potential antidepressive effect of ALCAR. In Papers 2 and 3, we demonstrated metabolic alterations in epileptic mice, such as glutamate reduction and mitochondrial dysfunction in the HF, partly resembling findings reported in human TLE. Moreover, we revealed that the turnover of important metabolites within and derived from tricarboxylic acid (TCA) cycle intermediates was decreased in both brain regions,

consistent with impaired function of the TCA cycle. ALCAR did not affect seizure development in PTZ kindled mice, but attenuated some metabolic alterations.

Altogether, the studies provide new information about metabolic alterations in the brain of epileptic mice and mice treated with ALCAR for 25 days. The findings may help improving diagnostics and treatment of TLE and evaluate the possible role of ALCAR in treating brain disorders.

The thesis is based on the following papers

Paper 1
Smeland O*, Meisingset T*, Borges K, Sonnewald U
Chronic acetyl-L-carnitine alters brain energy metabolism and increases noradrenaline and serotonin content in healthy mice
*Neurochemistry International, 2012 Jul, vol 61 (1) p 100-107, * Joint first authors*

Paper 2
Smeland O*, Meisingset T*, Sonnewald U
Dietary supplementation with acetyl-L-carnitine in seizure treatment of pentylenetetrazole kindled mice
*Neurochemistry International, 2012 Sep, vol 61 (4) p 444-454, * Joint first authors*

Paper 3
Smeland O, Hadera M, McDonald T, Sonnewald U, Borges K
Brain mitochondrial metabolic dysfunction and glutamate level reduction in the pilocarpine model of temporal lobe epilepsy in mice
Journal of Cerebral Blood Flow and Metabolism, 2013 Apr, In print

Other publications not included in this thesis

Walls A, Eyjolfsson E, Smeland O, Nilsen L, Schousboe I, Schousboe A, Sonnewald U, Waagepetersen H
Knockout of GAD65 has major impact on synaptic GABA synthesized from astrocyte-derived glutamine
Journal of Cerebral Blood Flow & Metabolism, 2011 Feb, vol 31 (2) p 494-503

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1. Introduction

Diseases of the brain, such as epilepsy, Alzheimer's disease and depressive disorders, have a large prevalence in the population. Not only do they affect the life quality of patients and their relatives, but they represent challenging issues for the society as a whole. Compared to the other life sciences, research on the brain has suffered from inadequate methods for investigating tissue function and pathogenesis, especially in the living human brain. Hence the understanding of many brain disorders is in general insufficient and treatment is not satisfactory. Indeed, acknowledging these challenges posed by brain diseases, President Barack Obama of the United States of America announced on April 2nd 2013 the BRAIN (Brain Research through Advancing Innovative Neurotechnologies) Initiative (WhiteHouse.gov 2013). The initiative is the largest research funding since the Human Genome Project with the preliminary goal to invent and refine new technologies to understand the human brain (Markoff and Gorman 2013).

The area of focus in this thesis is brain metabolism, in particular that of glucose, amino acids, energy and monoamines. In Paper 1, we aimed to investigate metabolic differences in the brain of mice supplemented with acetyl-l-carnitine (ALCAR), a potential therapeutic agent in the treatment of various brain disorders. In Paper 2, we evaluated the antiepileptic efficacy of ALCAR on seizures and metabolism in the pentylenetetrazole (PTZ)-kindling model of temporal lobe epilepsy (TLE) in mice. In Paper 3, we aimed to further elucidate metabolic alterations in another model of TLE in mice, namely the pilocarpine-status epilepticus (SE) model. The first part of this thesis serves as an introduction to basic concepts of brain metabolism, including the metabolic interplay between the major cell types of the brain, neurons and astrocytes, followed by a review about ALCAR metabolism. Thereafter, I give an introduction to epilepsy and TLE including the two animal models of TLE that were used in this thesis, and I discuss metabolic alterations in TLE. Next, I go through the different methodological procedures that we used, followed by summaries of the papers and finally the discussion of our work.

1.1. Cell types of the brain

By combining microscopy and various staining methods, researchers of the 19th century were able to demonstrate that biological tissue consists of individual cells. The procedures were inadequate for visualizing neural tissue, though, which lead to the common view that the brain was a net-like structure, i.e., a continuum of cells fused together (Langmoen and Apuzzo 2007). After the introduction of the Golgi silver-staining method, however, Ramón y Cajal in 1887 convincingly demonstrated that the brain consists of several distinct cells (Lopez-Munoz *et al* 2006). The pioneering work of Ramón y Cajal and others laid the framework for the Neuron Doctrine, the view that the elementary building blocks and signaling units of the brain

are individual neurons, which has been central for all disciplines in neuroscience since. The Norwegian explorer, diplomat and scientist Fridtjof Nansen was one of these pioneers in neuroscience. After completing his histological studies on neural tissue using the Golgi method, he stated in a paper dated 1886, that "no anastomoses were observed in the nervous systems of the organisms studied by me that could doubtlessly be identified as such" (Whiteley 2006).

The major cellular components in the brain are neurons and glia, the latter compromising astrocytes, microglia, radial glia, ependymal cells and oligodendroglia. Along with the microvessels of the brain, neurons and glia are organized into well-structured units capable of coordinating brain activity with local blood flow and blood-brain barrier functions (Abbott *et al* 2006). In this thesis, only neurons and astrocytes will be discussed, since these are the two main participants in brain metabolism. Neurons and glia differ widely in function and morphology, although they are derived from the same neuroepithelial cells of the embryonic nervous system. The traditional view has been that neurons are responsible for the primary function of the brain, information processing, whereas glia only support neuronal function. However, recent advances suggest a more active role of glia, as will be discussed below.

1.1.1. Neurons

Neurons are the primary signaling units of the nervous system that are organized in networks allowing simultaneous information processing in parallel systems. The complex operations require that neurons are able to communicate precisely and rapidly with each other over long distances. Three features give neurons these abilities: electrochemical excitability, asymmetric morphology and the production and release of neurotransmitters (Schwartz *et al* 2013). First, the fact that neurons are electrochemically excitable means that neurons may transform chemical or physical stimuli into transient electrical signals. This ability depends on the electrochemical difference across the cell membrane, the so-called membrane potential. The distribution of positively charged ions on either side of the cell membrane is at rest unequal, making the electrical voltage more negative inside than outside the cell. Various stimuli may alter the permeability of ions across the membrane via ion channels and receptors. If a certain threshold voltage is reached, an all-or-none electrical signal termed the action potential is generated. Importantly, the asymmetric morphology of neurons ensures unidirectional flow of signals. At one end, neurons have receptive dendrites that respond to chemical or physical stimuli via specific receptors. If an action potential is generated, it is conveyed to the other side of the neuron along the cellular process, the axon, terminating at synapses. Synapses are the sites of contact between a neuron and another cell and each neuron is innervated by thousands of synapses. The most common synapses are chemical, in which there is a separate cleft between the cells. Upon an action potential, vesicular neurotransmitters

are secreted into the synaptic cleft from the presynaptic neuron. The neurotransmitters cross the synapse and bind to specific receptors on the membrane of the postsynaptic neuron. Depending on the type of neurotransmitter and the expression of receptors at the post-synaptic membrane the binding elicit a specific response. Evidently, precise neurotransmission depends on a finely tuned system for the release and clearance of neurotransmitters from the synaptic cleft. Synapses may also be electrical by definition, in which gap junctions provide cytoplasmic continuity between cells.

The classical neurotransmitters include the amino acids glutamate, aspartate, γ-aminobutyric acid (GABA) and glycine, and the monoamines serotonin, dopamine and noradrenaline. Non-classical neurotransmitters include peptides (e.g. substance P), growth factors and gases (e.g. nitric oxide and carbon dioxide). The main excitatory neurotransmitter is glutamate and the main inhibitory is GABA. Both neurotransmitters bind to ionotropic and metabotropic receptors. Binding to ionotropic receptors rapidly opens ion gates, whereas binding to metabotropic receptors activates second-messenger pathways that influence ion channels in a slower fashion. Whereas glutamate and GABA is abundant throughout the brain, the monoamine neurotransmitters dopamine, serotonin and noradrenaline are synthesized only in small nuclei in the brain stem. However, they may influence the functions of several brain regions due to projections throughout the brain. Monoamines mainly act on membrane bound G-protein coupled receptors involving second-messenger pathways. There is a wide diversity of receptor isoforms and subtypes responding to the same neurotransmitter. Moreover, a neuron may respond to several neurotransmitters depending on the expression of receptors at the post-synaptic membranes. Thus, despite the relatively sparse number of neurotransmitters available, neurons produce diverse effects due to the variety of receptors and the integration of neurotransmission in space and time.

1.1.2. Astrocytes

Like neurons, the population of astrocytes is heterogeneous and can be distinguished on the basis of morphology, interactions with the local environment and biochemical, physiologic, and pharmacologic characteristics (Seifert *et al* 2006). Their name originates from their star-like shape upon Golgi staining, which is similarly reproduced by glial fibrillary acidic protein (GFAP) immunolabeling. However, it has become clear that GFAP immunostaining reveals only about 15% of the astrocytic domain since numerous fine processes do not express GFAP (Bushong *et al* 2002). Thus, the shape of astrocytes is more properly described as “spongiform”. Roughly, there are two main types of astrocytes: protoplasmic astrocytes with numerous fine processes found in gray matter and fibrous astrocytes with fewer processes found in white matter. Unlike neurons, astrocytes occupy separate domains that overlap minimally, only about 5% of the total astrocytic volume (Bushong *et al* 2002). Within their separate domains, end-feet dilatations on astrocytic processes cover capillaries

and arterioles, ensheathing more than 99% of the cerebrovascular surface (Takano *et al* 2006). In addition to covering microvasculature, end-feet of protoplasmic astrocytes envelop neurons and synapses whereas end-feet of fibrous astrocytes contact axons. Thus astrocytes are well positioned to link neuronal activity to blood flow and regulate the extracellular milieu. Moreover, astrocytic processes are coupled via gap junctions to form large intercellular networks allowing transport of small molecules such as K^+ or glutamate (Giaume *et al* 2010).

Astrocytes provide metabolic support to neurons and are critical for homeostasis of the neuronal microenvironment, including clearance of synaptic neurotransmitters and control of brain water homeostasis, extracellular K^+ concentration and local blood flow. Moreover, astrocytes synthesize and send neurotransmitter precursors to neurons, as will be discussed below. In the brain, K^+ buffering is coupled to the regulation of osmotic balance. The inwardly rectifying K^+ channel Kir4.1 and the water channel aquaporin-4 (AQP4) are colocalized primarily at the astrocytic perivascular endfeet (Nagelhus *et al* 2004). At the synapse, astrocytes clear excessive K^+ released by neuronal firing that could otherwise interfere with cell signaling and they may release K^+ at distant sites thus functioning as spatial buffers of K^+ (Wetherington *et al* 2008).

Although astrocytes are not electrically excitable, i.e., they cannot fire action potentials, several in vitro studies have demonstrated that astrocytes are chemically excitable and may modulate synaptic activity in the brain. Astrocytes possess functional neurotransmitter receptors and cultured astrocytes respond to glutamate with increases of cytosolic Ca^{2+} (Cornell-Bell *et al* 1990). Moreover, intracellular elevations of Ca^{2+} in astrocytes can result in the release of so-called gliotransmitters, notably glutamate, ATP and D-serine, which may modulate synaptic activity (Bezzi *et al* 1998; Jourdain *et al* 2007). Whether gliotransmission is a normally occurring event and astrocytes modulate synaptic transmission and plasticity in vivo, however, is still debated (Halassa and Haydon 2010; Hamilton and Attwell 2010).

1.2. Brain metabolism with emphasis on neuronal – astrocytic interactions

The brain is highly protected against physiological fluctuations in the external environment by the blood-brain barrier. The blood-brain barrier is formed by endothelial cells that line the microvessels of the brain restricting the passage of molecules due to tight junctions between adjacent cells. Within the brain, metabolism occurs in various compartments referring to the presence of functionally distinct pools of given metabolites that are not in rapid equilibrium with each other (Berl 1961). At the cellular level, the two main metabolic compartments in the brain are astrocytes and neurons. At the subcellular level, compartmentation may result from heterogeneity in the distribution of proteins, such as enzymes and transporters, or

from variations in morphology, internal physical barriers that limit diffusion, mitochondrial heterogeneity and so forth.

1.2.1. Glucose metabolism

The brain is an energetically expensive organ. Although the brain accounts for merely 2% of the body weight, it is responsible for up to 20% of resting body energy consumption (Attwell and Laughlin 2001). The high energy demand is related to cell signaling, including generation of action potentials, maintenance of ionic homeostasis and glutamate recycling. For the adult mammalian brain, glucose is the main energy substrate, whereas the developing brain has a higher usage of monocarboxylic acids such as lactate and ketogenic bodies. Driven by the higher glucose concentration in plasma than in brain interstitium, glucose crosses the blood-brain barrier and is taken up in cells through selective glucose transporters (Vannucci *et al* 1997). Although astrocytic endfeet cover almost the entire blood brain barrier, glucose diffuses paracellularly (Barros *et al* 2007) and evenly distributes between the intra- and extracellular space (Pfeuffer *et al* 2000). It has been estimated that neurons and astrocytes take up glucose at an approximately equal fraction (Nehlig *et al* 2004). However, it is shown that astrocytes use only 30% of the acetyl-CoA produced from glucose (Hassel *et al* 1995; Qu *et al* 2000). When in cytosol, glucose is rapidly metabolized to glucose-6-phosphate by hexokinase. Hexokinase activity is the rate-limiting step for glucose metabolism and the transport of glucose from plasma into cells is not saturated under physiological glucose concentrations (Barros *et al* 2007).

Glucose-6-phosphate has several possible metabolic fates. It is mainly metabolized through glycolysis to yield pyruvate, but may also be converted to glycogen to serve as energy storage or be funneled through the pentose phosphate pathway (PPP) (McKenna *et al* 2012). The PPP provides reduced nicotinamide adenine dinucleotide phosphate (NADPH) for maintenance of reduced glutathione and lipid biosynthesis as well as ribose-5-phosphate for nucleotide synthesis. In the cytosol, pyruvate may be reduced to lactate or transaminated to alanine. In mitochondria, pyruvate can be transaminated to alanine, carboxylated to oxaloacetate by the astrocytic specific enzyme pyruvate carboxylase (PC) (Patel 1974) or converted to acetyl-CoA by pyruvate dehydrogenase (PDH; in both neurons and astrocytes) (Hertz and Dienel 2002). Acetyl-CoA enters the tricarboxylic acid (TCA) cycle via condensation with oxaloacetate to form citrate, which is subsequently subjected to a cyclic series of oxidative steps. After one turn of the TCA cycle oxaloacetate is re-converted and may subsequently react with a new acetyl-CoA molecule and initiate another turn of the cycle. There is neither net synthesis nor net degradation of any TCA cycle intermediate during this operation and the sole purpose of pyruvate oxidation via acetyl-CoA is energy production. Through glycolysis and the TCA cycle, metabolism of glucose results in the reduction of electron carrier molecules nicotinamide adenine dinucleotide and flavin adenine dinucleotide. After reduction, these electron carrier molecules may transfer their electrons to O_2 at the

inner mitochondrial membrane in a series of reactions called the electron transport chain, driving the phosphorylation of adenosine diphosphate (ADP) to produce adenosine-5'-triphosphate (ATP). Complete oxidation of one molecule of glucose requires 6 oxygen molecules and yields enough chemical energy to produce a maximum of 38 ATP molecules, according to the following equation: $C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$. Under basal physiological conditions, glucose is mainly oxidatively metabolized, compatible with an oxygen/glucose index for glucose metabolism close to 6 (Madsen et al 1999). Recently it was estimated that energy requirements of signaling and non-signaling events in the brain are constant and independent of signaling frequency (Hyder *et al* 2013). The results indicate that there is a linear relationship between brain activity levels and increases in blood flow, blood volume and oxygen consumption. Moreover, the authors showed that the energy requirements are conserved across mammalian species suggesting that early in the evolution the mammalian brain optimized the balance between energy consumption and information processing at the cellular level.

1.2.2. Amino acid metabolism

Amino acid neurotransmitter metabolism is linked to glucose metabolism via the TCA cycle intermediates α-ketoglutarate and oxaloacetate. The main excitatory neurotransmitter glutamate is synthesized from α-ketoglutarate by glutamic acid dehydrogenase or various aminotransferases. Glutamate is either packaged into vesicles for neurotransmitter use or added to the pool of metabolic constituents. After vesicular release into the synapse glutamate is predominantly taken up by astrocytes through excitatory amino acid transporters 1 and 2, maintaining extracellular glutamate concentrations thousand-fold lower than inside cells (Danbolt 2001). Rapid clearance of glutamate ensures high signal to noise ratio for neurotransmission and avoids cell damage due to excessive stimulation (excitotoxicity). When in astrocytes, glutamate may enter the TCA cycle through conversion to α-ketoglutarate or be converted to the non-excitatory compound glutamine by the astrocyte-specific enzyme glutamine synthetase (GS) (Norenberg and Martinez-Hernandez 1979). Glutamine in turn is exported and taken up by neurons, where it is deamidated to glutamate by phosphate-activated glutaminase, completing the so-called glutamate-glutamine cycle (Kvamme *et al* 1988; van den Berg and Garfinkel 1971). The cycle serves as a buffer reservoir of the precursor glutamine for the synthesis of glutamate (and GABA) and also functions as a ammonia detoxification system in the brain. The glutamate–glutamine cycle does not operate stoichimetrically since some glutamate is converted to α-ketoglutarate and metabolized in the TCA cycle in both neurons and astrocytes.

GABA is synthesized by decarboxylation from glutamate by glutamic acid decarboxylase. Neuronal GABA is either packaged into vesicles for synaptic release or enters the TCA cycle after conversion to succinic acid semialdehyde and then

succinate by the concerted action of GABA transaminase and succinate semialdehyde dehydrogenase (Balazs *et al* 1970). Unlike glutamate, most GABA is taken up by the pre-synaptic neuron and to a lesser extent by the astrocyte (Roberts and Frankel 1950; Schousboe *et al* 2004). Thus GABAergic neurons depend to a lesser degree than glutamatergic neurons on astrocytic glutamine, although a GABA-glutamate-glutamine cycle is still present. See Figure 1 for an overview of the metabolism of glutamate, glutamine and GABA in neurons and astrocytes.

Loss of TCA cycle intermediates occurs via various enzymatic reactions such as the conversion of α-ketoglutarate to glutamate. Since not all glutamate released from neurons is fully recycled from astrocytes and the blood brain barrier is poorly permeable for glutamate and glutamine, there is a need for new synthesis of TCA cycle intermediates to maintain energy and amino acid production. The formation of deficient TCA cycle intermediates from precursors that are not TCA cycle intermediates themselves is called anaplerosis. The main anaplerotic enzyme in the brain is PC (Patel 1974), which is localized in astrocytes only (Yu *et al* 1983). PC combines CO_2 and pyruvate leading to net synthesis of oxaloacetate. Thus neurons depend on re-filling of their TCA cycle by newly synthesized glutamine from astrocytes to maintain the synaptic glutamate pool. Other anaplerotic pathways include the conversion of pyruvate to malate by malic enzyme, although it operates predominantly in the opposite direction in the brain (Patel 1974).

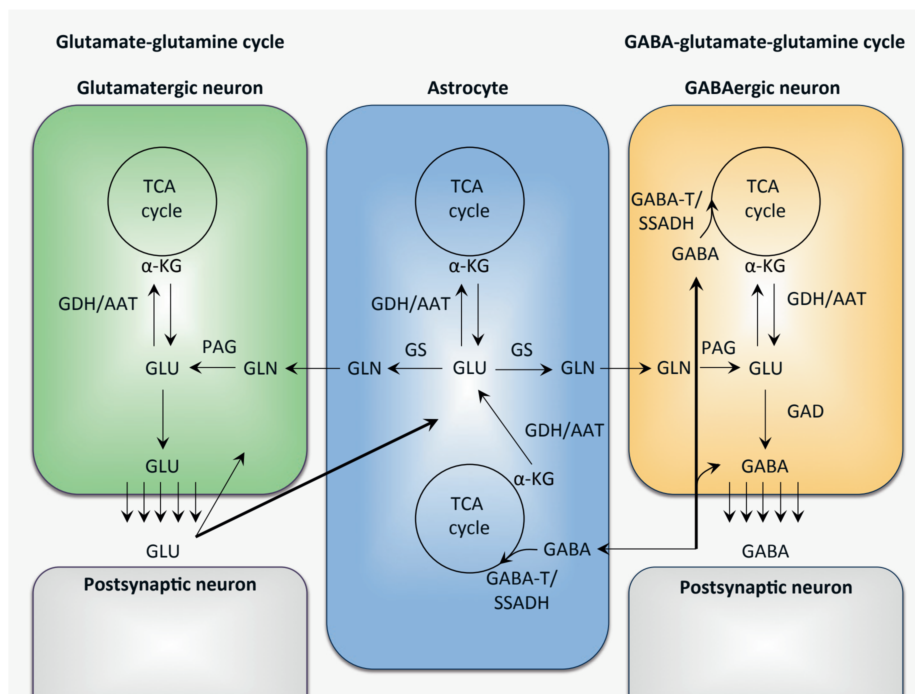

Figure 1 Simplified schematic representation of the metabolism of glutamate (GLU), glutamine (GLN) and GABA in neurons and astrocytes. GLU is the precursor for GABA and GLN. GLU is formed from α-ketoglutarate (α-KG) via either glutamate dehydrogenase (GDH) or aspartate aminotransferase (AAT). After vesicular release from glutamatergic neurons, synaptic glutamate is predominantly taken up by astrocytes. Here glutamate can be converted to glutamine (GLN) by glutamine synthetase (GS) or enter the tricarboxylic acid (TCA) cycle via α-KG. GLN is subsequently transported back to neurons and reconverted to GLU by phosphate-activated glutaminase (PAG). GLU is then packaged into vesicles or enter the TCA cycle. In GABAergic neurons, GABA is produced from GLU by glutamate decarboxylase (GAD). Synaptic GABA is predominantly taken up by neurons. After uptake in neurons, GABA may be repackaged into vesicles or enter the TCA cycle via the sequential action of GABA transaminase (GABA-T) and succinate semialdehyde dehydrogenase (SSADH). In astrocytes, GABA enters the TCA cycle via the GABA-T/SSADH pathway. Subsequently, the TCA cycle may give rise to glutamate, which is converted to glutamine and transported back to the GABAergic neuron, whereupon GABA is formed from glutamate. Adapted from McKenna et al (2012).

1.3. ALCAR

ALCAR is a common dietary supplement readily available in health food stores abroad but not in Norway, claimed to improve energy levels and muscle strength. Due to its high tolerability and mild adverse effects in humans, supplementation with ALCAR is favorable as medical treatment and has been tried out in various brain disorders for more than two decades. There is now evidence for beneficial effects of ALCAR in the treatment of patients with depressive disorders and related conditions, although the trials have been carried out on small groups only (Martinotti *et al* 2011; Pettegrew and McClure 2002; Soczynska *et al* 2008; Zanardi and Smeraldi 2006). The results from trials with Alzheimer's disease have been conflicting, and a Cochrane metaanalysis found no convincing effects (Hudson and Tabet 2003). Moreover, ALCAR is marketed for the treatment of peripheral neuropathies associated with diabetes and HIV (Evans *et al* 2008; Hart *et al* 2004; Youle *et al* 2007).

ALCAR is the short-chain ester of l-carnitine, an endogenous compound present in all mammalian tissue (Bremer 1983). L-carnitine is mainly acquired through the diet, but may also be synthesized in vivo from the precursors lysine and methionine, mostly in the liver and kidney (Rebouche 2004). The main physiological role of the carnitine system is to facilitate ß-oxidation by transporting acyl-moieties from fatty acids across the mitochondrial membrane (Bartlett and Eaton 2004). Although the brain mainly relies on glucose as energy substrate, the carnitine system is present in the brain and metabolism of fatty acids can account for 20% of oxidative brain energy production in adult rats (Ebert *et al* 2003).

The concentrations of ALCAR in plasma and CSF increase after oral administration, and the compound is easily transported across the blood-brain barrier primarily via the organic cation/carnitine transporter OCTN2 (Kido *et al* 2001; Parnetti *et al* 1992). Moreover, carnitine transporters from the OCTN family are present on both neurons and astrocytes (Januszewicz *et al* 2009; Januszewicz *et al* 2010). The reversible transfer of the acetyl moiety from carnitine to free CoA is catalyzed by the enzyme carnitine acetyltransferase, which is located on the inner mitochondrial membrane as well as in the endoplasmic reticulum and peroxisomes (Bieber 1988). See Figure 2 for a simplified overview of ALCAR metabolism in relation to glucose metabolism in a cell.

The metabolic fate of ALCAR has been thoroughly investigated in the rodent brain. The acetyl moiety of ALCAR readily enters the acetyl-CoA pool (Jones *et al* 2010), and 60% of radioactivity from [1-^{14}C]ALCAR injected into the lateral rat brain ventricle was recovered as $^{14}CO_2$ in the expirium, indicating oxidative metabolism in the TCA cycle (Ricciolini *et al* 1998). The majority of the remaining radiolabel in the brain was incorporated in phospholipids. Recently, Scafidi et al. (2010a) combined intraperitoneal injection of [2-^{13}C]ALCAR with ex vivo nuclear magnetic resonance (NMR) spectroscopy in juvenile rats. The authors demonstrated that the acetyl moiety is utilized for the synthesis of amino acid neurotransmitters via both the neuronal and

astrocytic compartments. The acetyl-moiety of ALCAR may also be utilized for the synthesis of acetylcholine (Dolezal and Tucek 1981).

ALCAR treatment has pronounced effects on cerebral glucose and energy metabolism. In rats, autoradiography revealed increased [^{14}C]2-deoxyglucose labeling in several brain regions after both single and chronic ALCAR administration (Freo *et al* 2009; Ori *et al* 2002). Single injection of ALCAR reduced the oxidation of glucose and stimulated the synthesis of glycogen (Aureli *et al* 1998), and increased the amount of phosphocreatine and reduced the levels of lactate and free organic phosphate in the rat brain (Aureli *et al* 1990). Moreover, ALCAR improved the neurological outcome in various animal models of ischemia accompanied by restoration of aerobic energy metabolism, underlining the positive effects of ALCAR on brain energy homeostasis (Aureli *et al* 1994; Rosenthal *et al* 1992; Scafidi *et al* 2010b).

Single administration of ALCAR affects numerous neurotransmitter systems in the rat brain (Tempesta *et al* 1985; Toth *et al* 1993). Single administration of ALCAR before injection of 3,4-methylenedioxymethamphetamine (ecstasy) to rats prevented loss of serotonin in various brain regions (Alves *et al* 2009). In controls, the only effect of ALCAR pretreatment was increased content of the serotonin metabolite 5-hydroxyindoleacetic acid, but not serotonin, in one of six investigated brain regions, namely the prefrontal cortex. In a model of attention deficit hyperactive disorder, chronic ALCAR treatment normalized the level of noradrenaline and the serotonin turnover ratio in specific brain regions (Adriani *et al* 2004). In normal young rats the only change was an increased serotonin turnover ratio in the cingulate cortex.

Moreover, the acetyl-moiety of ALCAR is readily available for transacetylation of functional groups on amino acids, which could potentially modify protein structure and activity (Pettegrew *et al* 2000). Other neurobiological effects of ALCAR include modulation of membrane composition and neurotrophic factors (Jones *et al* 2010; Pettegrew *et al* 2000). Finally, ALCAR protects against neurotoxic insults such as exposure to glutamate and amyloid-ß, and has both antioxidant and anti-apoptotic properties (Forloni *et al* 1994; Ishii *et al* 2000; Liu *et al* 1993).

In conclusion, the effects of exogenously administered ALCAR on brain metabolism have been extensively studied, but mostly in rats after single exposure. The acetyl-moiety of ALCAR readily enters the acetyl-CoA pool and may be utilized for the generation of energy and synthesis of neurotransmitters. Moreover, single administration of ALCAR has been shown to alter the content and release of various neurotransmitters in several brain regions.

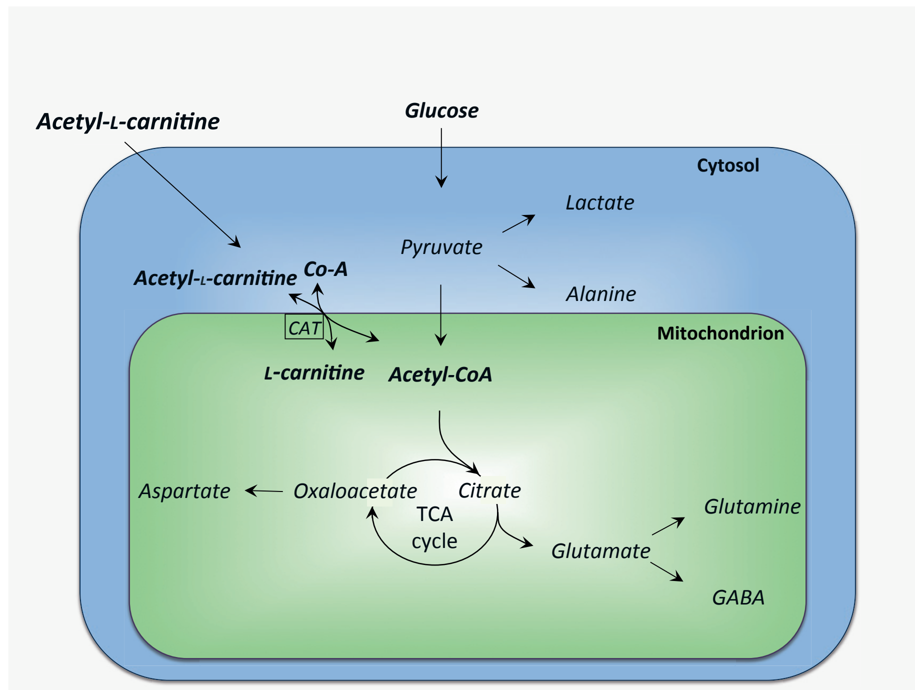

Figure 2 Simplified overview of acetyl-l-carnitine (ALCAR) metabolism in relation to glucose metabolism in a cell. The reversible transfer of the acetyl moiety from carnitine to free CoA is catalyzed by the enzyme carnitine acetyltransferase (CAT) located on the inner mitochondrial membrane. Acetyl-CoA from ALCAR enters the acetyl-CoA pool and can be incorporated into the TCA cycle.

2. Epilepsy

Epilepsy is the second most common neurological disorder after stroke (Porter 1993), affecting about 65 million persons worldwide (Thurman *et al* 2011). As early as about 400 BC it was proposed in the Hippocratic writings that seizures originates from the brain, but the link was not properly established until the mid 19th century by John Hughlings Jackson (Eadie 1995). Since then epileptology has endured a rapid development in parallel with seminal discoveries in neuroscience.

Epilepsy is a disorder of the brain characterized by repetitively occurring seizures (Fisher *et al* 2005). The disorder includes several subtypes with distinct phenotypes and pathophysiologies that are classified by etiology into symptomatic (secondary to another disease, e.g. cancer or brain insult), idiopathic (arises from itself) and cryptogenic (undetermined etiology, probably symptomatic) (Engel 1996). Basically, the epileptic brain exists in two functional states: the ictal state during a seizure and the longer interictal state in between seizures. The biological basis for the transition from the interictal state to a seizure is termed ictogenesis, whereas epileptogenesis refers to the underlying processes leading to the development of the chronic phase of epilepsy with spontaneous recurrent seizures (Pitkanen and Lukasiuk 2011). Neither ictogenesis nor epileptogenesis are well understood today. Current epilepsy treatment strategies largely aim at decreasing neuronal excitability and thereby preventing the occurrence of seizures. An alternative approach is the targeting of epileptogenesis preventing the emergence of the epileptic state. Currently no effective antiepileptogenic treatments are available. Approximately one in three epilepsy patients are pharmacoresistant and suffer from uncontrollable seizures, emphasizing the need for newer treatment strategies (Kwan and Brodie 2000). The consequences of uncontrollable epilepsy can be severe including social disability, neuropsychological impairment and increased morbidity and mortality (Sperling 2004).

Seizures arise from an area termed the epileptic focus and are classified as partial, meaning onset in one hemisphere, or generalized, involving both hemispheres, based on clinical findings and electroencephalography (Engel 2006). If consciousness is altered seizures are described as complex and simple if otherwise. Since epileptic seizures are transient disruptions in brain function, the signs and symptoms of seizures depend on the localization and function of the affected brain area and the degree of spread to other areas. Previously, epileptic seizures have been thought to result from massive hypersynchronous activity due to hyperexcitability in large numbers of neurons in the brain (Fisher *et al* 2005). However, a recent study demonstrated that neuronal spiking behavior is highly heterogeneous, not hypersynchronous, during seizure initiation, indicating complex interplay among subsets of neurons (Truccolo *et al* 2011).

2.1. TLE

TLE is the most common form of focal epilepsies and likely the most common form of human epilepsies (Engel and Williamson 2008). There are two main types of TLE, mesial and lateral. In mesial TLE (MTLE), seizures arise from the inner aspect of the temporal lobe, particularly the hippocampus. In lateral TLE, seizures arise from the cortical layer on the outer surface of the temporal lobe and may be caused by tumors, vascular malformations or cortical dysplasias, among others. MTLE accounts for two-thirds of the TLEs (Panayiotopoulos 2012). Seizures of MTLE typically begin with an aura, a simple partial seizure that may progress with motor and sensory signs and loss of consciousness (Engel 2001) (the experience of an aura preceding the loss of consciousness during an epileptic seizure is vividly described by Fyodor M. Dostoyevsky in an excerpt from the novel "The Idiot" reproduced in the opening pages of this thesis).

Patients with TLE often have a history of an initial precipitating injury (IPI) that occurred within the first 4 or 5 years of life (Mathern *et al* 2002). An IPI is any clinically significant medical event with neurological implications occurring prior to epilepsy. The most common IPI is complex febrile seizures associated with 67% of patients with TLE (French *et al* 1993). However, since febrile seizures are common in the general population, they rarely signify the development of epilepsy, suggesting that genetic susceptibility plays a major role in epileptogenesis (Cross 2012). Other important IPIs are head trauma, intracerebral infections and SE. Typically there is a latency period of a mean 8-9 years between an initial precipitating injury and the emergence of spontaneous recurrent seizures (i.e., the epileptogenic period) (Mathern *et al* 2002). As mentioned above, there is no coherent explanation to epileptogenesis, and mechanisms include inflammation, neurodegeneration, alterations in the function and expression of ion channels and receptors, as well as blood-brain-barrier disruption (Pitkanen and Lukasiuk 2011).

After the first ictal event, patients with TLE usually respond well to antiepileptic drugs. However, as many as 60% of patients become pharmacoresistant during the course of their condition, in contrast to 20% of patients with primary generalized epilepsy (Pati and Alexopoulos 2010). Despite the high degree of drug-resistance associated with TLE, the condition is effectively treated by surgical resection (Engel 2001). The outcome of surgery highly depends on the ability to precisely localize the epileptic focus, using non-invasive imaging techniques such as magnetic resonance imaging and positron emission tomography. The imaging methods for mapping the epileptic brain and the availability of resected human seizure foci have been invaluable tools for studying the pathophysiology of TLE.

A hallmark of hippocampal tissue from TLE patients is sclerosis. Hippocampal sclerosis is usually unilateral and refers to loss of neurons, gliosis and network reorganization (Blumcke *et al* 2002). Neuronal loss is typically segmental, affecting pyramidal neurons in the CA1, CA3 and CA4 regions of the hippocampal formation (HF). The gliosis that accompanies neuronal loss is characterized by hypertrophy of

astrocytic cell bodies and processes with altered domain organization and increased expression of GFAP rather than an increase in astrocyte number (Oberheim *et al* 2008). Moreover, there is axonal and synaptic reorganization such as sprouting of axons (mossy fibers) of dentate granule cells that may contribute to hyperexcitable circuits. Whether hippocampal sclerosis merely is a consequence of seizures or if it represents pre-existing hippocampal abnormalities that dispose to TLE remains uncertain. Similar lesions can be found in extrahippocampal structures such as the parahippocampal cortices, amygdala and thalamus (Chassoux *et al* 2004).

2.2. Animal models of epilepsy

The use of animal models for reproducing human diseases has played a vital role in modern experimental medicine. This has certainly been the case for the research on epilepsy, in which animal model studies have contributed vastly to the understanding of pathophysiological processes and the development of antiepileptic treatment strategies. A wide diversity of models of epilepsy and seizures exists, ranging from invertebrate models such as drosophila to non-human primates. Models may be induced chemically or electrically or result from genetic manipulation (Sarkisian 2001). The animal models are characterized into models of seizures or models of epilepsy with spontaneously recurrent seizures. Seizure models have been pivotal for the discovery and development of antiepileptic drugs and for studying seizure mechanisms. Models of epilepsy, on the other hand, better represent the human form and offer the opportunity for studying epileptogenesis and the morphological and functional disturbances present in the chronic phase of epilepsy. There has been a growing concern that acute seizure models may fail to predict efficacy of antiepileptic drugs, at least in cases of pharmacoresistant epilepsy and that other models of epilepsy should be used more extensively (Loscher 2011).

For animal models to be applicable to humans there are three criteria that must be fulfilled (Coenen and Van Luijtelaar 2003): 1) Face validity: that the behavior and appearance of the model and the modeled disorder are similar; 2) Predictive validity: that testing of clinically established drugs in the model elicits similar responses as in humans. Thus the probability of the model to predict therapeutic responses increases; 3) Construct validity: that the etiological, neurophysiological, neuropathological and genetic hypotheses of the disorder correspond to the theoretical basis of the model.

The characteristics of TLE can be reproduced in chronic animal models, in particular kindling and post-SE animal models. In this thesis, we used a PTZ kindling model (Paper 2) and a pilocarpine-SE model (Paper 3).

2.2.1. The PTZ kindling model

Kindling is the phenomenon by which repeated application of subconvulsive stimuli leads to an incremental increase in seizure susceptibility (Sarkisian 2001). Kindling can be induced electrically or chemically and is commonly viewed as a model of epileptogenesis and TLE (Bertram 2007; Morimoto *et al* 2004). The duration, length and severity of seizures increase gradually in parallel with decreased seizure threshold until a plateau is reached. The change in seizure susceptibility is permanent and general, meaning that animals already kindled chemically or electrically develop seizures faster and with increased severity if kindled using another agent compared to animals not previously kindled (Cain 1981; Cain 1982). This phenomenon is called transfer and indicates that electrical and chemical kindling influence similar seizure circuitry.

PTZ was first described as a chemoconvulsant in 1926 (Hildebrandt 1926). The compound has been used to generate models of acute seizures (Green and Murray 1989), the kindling model (Mason and Cooper 1972) and models of chronic convulsive seizures (Cremer *et al* 2009). PTZ appears to mediate its effect through binding to the picrotoxin-binding site of the post-synaptic $GABA_A$ receptor, which may lead to desensitization of the GABA receptor (Kamphuis *et al* 1990; Macdonald and Barker 1978).

In terms of face validity, the clinical picture of kindling mimics complex partial seizures with secondary generalization. Due to cortical involvement it has been argued that kindling is closer to human primary generalized epilepsy than TLE (Gilbert and Goodman 2006). Kindled animals can develop spontaneous recurrent seizures if they are kindled for a prolonged time, although this is not often done (Sarkisian 2001). In terms of construct validity, the kindling model does not display a latency period, in contrast to that seen in humans after an IPI. It can, however, be considered an advantage that epileptogenesis is modeled in a more standard and controlled manner in kindling models than in post-SE models (McIntyre *et al* 2002). In terms of construct validity, PTZ kindling mimics TLE by inducing sprouting and synaptic reorganization (Cavazos *et al* 2004; Golarai *et al* 1992) including neuronal loss and gliosis in the hippocampus (Franke and Kittner 2001), although the extent of cell death is less compared to post-SE models (Sarkisian 2001). With regard to predictive validity, the PTZ kindling model is considered a sensitive screening tool for antiepileptic drug efficacy, and identified the effects of levetiracetam, which was missed using standard seizure models (Klitgaard *et al* 1998).

2.2.2. The pilocarpine-SE model

In rodents, systemic or intracerebral administration of the cholinergic agonist pilocarpine induces SE with the subsequent appearance of spontaneous recurrent seizures and neuropathological changes resembling human TLE (Turski *et al* 1989).

Along with the kainate-induced SE model, the pilocarpine-SE model is probably the most commonly studied chemically induced model of TLE (Sarkisian 2001). In terms of construct validity, the SE model complies with the etiological hypothesis of TLE that an initial injury precipitates the development of the disorder.

Pilocarpine induces SE through activation of muscarinic receptors rapidly followed by an imbalance between excitatory and inhibitory transmission probably mediated by glutamatergic responses (Priel and Albuquerque 2002). Pilocarpine-SE can be blocked by systemic administration of the muscarinic antagonist atropine, but atropine has no effect once seizures are initiated (Clifford *et al* 1987). After SE, there is usually a latency period from days to weeks before the emergence of spontaneous recurrent seizures (Cavalheiro 1995). The seizures can be classified as partial with or without secondary generalization. Electrographically the seizures are characterized by paroxysmal hippocampal discharges that rapidly spread to cortical areas (Cavalheiro *et al* 1991). Thus the model complies with criteria for face validity by producing limbic seizures in coherence with those seen in TLE.

Histopathologically, pilocarpine-SE animals share similar phenomena as TLE patients (i.e., construct validity). There is extensive neuronal death in the hippocampus and adjacent limbic structures accompanied by mossy fiber sprouting, synaptic reorganization and gliosis (Borges *et al* 2003; Motte *et al* 1998). In terms of predictive validity, seizures in the pilocarpine-SE model show similar response to antiepileptic drugs as in human TLE (Chakir *et al* 2006; Glien *et al* 2002). It is considered a weakness that the model can only be induced in adult animals, since administration of pilocarpine to young rodents (<P20) does not lead to spontaneous recurrent seizures and associated pathological changes (Sarkisian 2001).

2.2.3. Metabolic alterations in TLE

Although neuronal activity may be heterogeneous at the onset of seizures and not hypersynchronous as previously believed (Truccolo *et al* 2011), seizures fundamentally result from an imbalance between too much excitation and too little inhibition. In line with this, currently available antiepileptic drugs are thought to decrease excitatory neurotransmission and enhance inhibitory by targeting molecules at the excitatory and inhibitory synapses (Bialer and White 2010). However, the metabolic picture seen in epileptic tissue is not as straightforward as an imbalance between too much glutamate and too little GABA.

A large number of studies in animals and humans have shown that glutamate and glutamate analogues, such as kainic acid and domoic acid, can cause seizures and neuronal loss similar to TLE (Ben-Ari 1985; Olney *et al* 1972; Teitelbaum *et al* 1990). In patients with TLE, in vivo microdialysis revealed 6-fold increase in levels of extracellular glutamate within the epileptogenic hippocampus before and during seizures (During and Spencer 1993). Interictally, extracellular glutamate was 5-fold higher in epileptogenic versus the nonepileptogenic hippocampi (Cavus *et al* 2005).

Moreover, the extracellular glutamate concentration remained markedly elevated for at least 20 min after the cessation of seizure activity (During and Spencer 1993), suggesting decreased clearance of glutamate from the synapse. Whether uptake of glutamate via astrocytic glutamate transporters is dysfunctional in the epileptogenic hippocampus remains controversial (Bjornsen *et al* 2007). Interestingly, the tissue content of glutamate was decreased in the epileptogenic versus normal hippocampi in patients with TLE, which did not correlate with the extent of neuronal loss (Petroff *et al* 2002b). In epilepsy patients undergoing mesial temporal lobe resection, [2-^{13}C]glucose was infused in vivo and ^{13}C NMR spectroscopy was performed on brain tissue extracts to analyze the resected hippocampi (Petroff *et al* 2002a). The work demonstrated that the rate of glutamate-glutamine cycling in comparison to the overall TCA cycle was decreased in patients with hippocampal sclerosis compared to normal hippocampi. In another group of patients with TLE, the expression of the astrocytic enzyme GS was decreased in the epileptogenic hippocampi, suggesting reduced conversion of glutamate to glutamine in astrocytes which could explain the accumulation of synaptic glutamate (Eid *et al* 2004; van der Hel *et al* 2005). Since the glutamate-glutamine cycle is a major contributor to vesicular GABA, downregulation of GS could also impair GABAergic inhibition (Liang *et al* 2006). In two post-SE models of TLE in rats investigated by our group, ^{1}H NMR spectroscopy revealed decreased hippocampal concentrations of glutamate but normal concentrations of GABA (Alvestad *et al* 2008; Melø *et al* 2005). The models also displayed alterations in the contents of glutamate, GABA and aspartate in cortical regions.

The level of extracellular GABA was higher during and after seizures in both epileptogenic and nonepileptogenic hippocampi in patients with TLE (During and Spencer 1993). Interestingly, GABA concentrations were higher in the nonepileptogenic hippocampus compared to the epileptogenic. The role of GABA in epilepsy is complicated with the demonstration that GABA in some settings may be excitatory (Kohling 2002). An in vitro study of brain tissue from patients with TLE showed that GABA released by interneurons depolarized excitatory pyramidal neurons likely to initiate epileptic discharges (Cohen *et al* 2002). Moreover, spontaneous rhythmic activity in brain slices of human epileptic hippocampi appeared to be mediated by GABAergic mechanisms (Schwartzkroin and Haglund 1986). On the other hand, inhibition of the TCA cycle of GABAergic neurons using 3-nitropropionic acid induced seizures in mice (Hassel and Sonnewald 1995).

During seizure initiation and propagation there are dramatic increases in cerebral blood flow and metabolism in the epileptic focus (Kobayashi *et al* 2006). In contrast, functional neuroimaging methods demonstrate hypometabolism of glucose in seizure foci and adjacent brain structures interictally (Engel *et al* 1982; O'Brien *et al* 1997). Neuronal loss does not appear to correlate with the decrease in glucose consumption, suggesting that interictal hypometabolism results from dysfunctional mitochondrial oxidative and/or glycolytic energy metabolism in epileptic tissue (Dube *et al* 2001; O'Brien *et al* 1997). In line with this, decreased content of the neuronal marker *N*-acetyl aspartate (NAA) is reported in the hippocampi of animals and patients with TLE (Alvestad *et al* 2008; Cendes *et al* 1994; Melø *et al* 2005). There seems to be no

significant relationship between content of NAA and cell death suggesting that NAA loss reflects neuronal metabolic dysfunction rather than neuronal loss (Cohen-Gadol *et al* 2004; Kuzniecky *et al* 2001; Petroff *et al* 2002b; Vielhaber *et al* 2008). Moreover, Kann et al. (2005) used fluorescence recordings of nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) to evaluate the status of energy metabolism in hippocampal slices from TLE patients. The recordings revealed alterations in transients of NAD(P)H during neuronal activation compatible with an impairment of mitochondrial function (Kann *et al* 2005). Mitochondrial oxidative stress might also be an important contributor to epileptogenesis and reactive oxygen species are integral to processes such as excitotoxicity and apoptosis that contribute to seizure-induced brain damage (Waldbaum and Patel 2010).

In addition to displaying altered activity of enzymes such as GS, astrocytes may contribute to epileptoform activity by releasing glutamate via calcium signaling and depolarize local neurons (Tian *et al* 2005). Interestingly, the ability of astrocytes to transmit calcium signaling was reduced by several antiepileptic drugs, including valproate, gabapentin and phenytoin. Moreover, several lines of evidence suggest impaired K^+ redistribution by astrocytes in epileptic tissue that could lower seizure threshold and increase likelihood of seizures. As previously described, K^+ buffering in the brain is coupled to water flux and there is loss of AQP4 channels from astrocytic perivascular endfeet in sclerotic hippocampi from patients with TLE (Eid *et al* 2005). Impaired water transport could also lead to swelling of astrocytes in epileptic tissue, reducing the extracellular space and strengthening the electric field among tightly packed neurons (Wetherington *et al* 2008). Furthermore, astrocytes may affect astrocytic glutamate signaling by releasing inflammatory molecules such as tumor necrosis factor α (Wetherington *et al* 2008).

In conclusion, altered interplay between GABAergic and glutamatergic neurotransmission involving both neurons and astrocytes seems to be vital for generating epileptic seizures. Additionally, a considerable number of studies argue for an underlying metabolic dysfunction not correlated with neuronal death as an important pathophysiological mechanism of TLE. Moreover, disturbed astrocytic functions may have implications for epilepsy.

3. Methods

3.1. Experimental procedures with animals

In all studies mice were used. The animals had free access to food and water and were housed in individual cages with a 12-hour light-dark cycle. The animals were adapted to these conditions for at least 1 week before being used in the experiments and weight and general condition were monitored throughout the study. The animal experiments were approved by the local ethic committees and were in accordance with national and international guidelines. All efforts were made to minimize the suffering and the number of animals in line with modern principles of animal research, notably the three R's (replacement, reduction, refinement) (Russell and Burch 1959). For experimentation, the animals were randomly divided in groups and the investigators were blinded to their treatment.

3.1.1. Papers 1 and 2

Fifty male 4-week-old NMRI mice with an average weight of 25 g were divided in 5 groups: control, ALCAR-control, PTZ-control, PTZ-ALCAR and PTZ-valproate. The two experiments were carried out simultaneously using the same control group.

3.1.1.1. ALCAR and valproate supplementation

In Papers 1 and 2, ALCAR and valproate were supplemented in the drinking water for 4 days before the experimental procedure with PTZ started and continued throughout the experiments. Mice subjected to ALCAR treatment received a 1.5 g/L solution of ALCAR as drinking water and were allowed to drink ad libitum. ALCAR-control mice drank an average of 9.9 ± 0.6 ml per day (calculated by weighing the water bottles), which provides a daily ALCAR dose of 496 ± 21 mg/kg body weight. PTZ kindled mice supplemented with ALCAR drank an average of 10.1 ± 1.1 ml per day, which provides a daily ALCAR dose of 506 ± 36 mg/kg body weight. These doses are in accordance with effective ALCAR regimens in previous studies (Hagen *et al* 2002; Mollica *et al* 2001). In Paper 2, a group of PTZ kindled mice was subjected to valproate treatment as positive control. The group received a 3.3 g/L solution of valproate as drinking water and was allowed to drink ad libitum. The mice drank an average of 8.4 ± 1.4 ml per day, which would provide a daily valproate dose of 920 ± 48 mg/kg body weight. Healthy control mice drank an average of 7.8 ± 1.2 ml per day, whereas PTZ-control mice drank an average of 7.5 ± 1.6 ml water per day.

At the end of the study, the mean weights of the groups were: control mice: 36.5 ± 0.7 g; ALCAR mice: 36.6 ± 1.0 g; PTZ-control mice: 34.2 ± 1.6 g; PTZ–ALCAR

mice: 35.7 ± 1.1 g; and PTZ–valproate mice: 32.1 ± 0.7 g. The PTZ–valproate mice weighed significantly lower than control mice and PTZ–ALCAR mice, when comparing the four groups in Paper 2.

3.1.1.2. PTZ kindling

PTZ kindling was induced using a similar experimental protocol as Hansen et al. (2009). Mice were injected with PTZ (43 mg/kg i.p.) three times a week for three weeks. After PTZ injection mice were observed for 30 min for latency to clonic convulsions and the severity of convulsions was scored according to a modified Racine scale (0 = no response, 1 = 1–3 myoclonic jerks and/or facial twitching and/or axial waves going though the body, 2 = more than 3 myoclonic jerks, 3 = clonic convulsion with forelimb clonus without loss of postural control, 4 = clonic convulsion with loss of postural control, turning to the side and/or rearing, 5 = clonic convulsion with loss of righting reflex and/or bouncing, two or more clonic convulsions, tonic convulsion or SE) (Hansen *et al* 2009). The mice were randomized before PTZ injections and the observers were blinded to the identity of the animal.

In the PTZ-control group one mouse died from SE, and one was found moribund. One mouse died in the PTZ–ALCAR group during SE. Before the end of the study, 3 mice from each PTZ-group and the control group were withdrawn from further experimentation, as they were intended to be part of another study.

3.1.2. Paper 3

7-8-week-old male CD1 mice were used. They were divided in two groups: controls and pilocarpine-SE mice.

3.1.2.1. The pilocarpine model

15-30 minutes prior to pilocarpine (330-345 mg/kg s.c. in 0.9% saline) injection, 35 CD1 mice were injected with methylscopalamine (2 mg/kg i.p. in 0.9% NaCl). Methylscopalamine is a cholinergic antagonist with limited permeability across the blood-brain barrier. Thus, pre-treatment with methylscopalamine minimizes peripheral side effects of pilocarpine administration. Twenty-three pilocarpine injected mice (66%) experienced behavioral SE as defined by continuous seizure activity consisting mainly of whole body continuous clonic seizures, while nine mice died and three showed no SE. Only mice exhibiting SE were used further in the studies. Ninety minutes after pilocarpine administration, SE was terminated with injection of pentobarbital (22.5 mg/kg i.p. in 0.9% NaCl) followed by 1 ml 4% dextrose in 0.18% saline (s.c.).

All mice were hand-fed moistened cookies and injected with 5% dextrose in lactate Ringer's solution twice a day for about three days and thereafter when needed. No systematic observations were done to detect spontaneous seizures in mice, but 21 mice with SE were observed to have handling induced seizures and altered behavior, including lack of nest building and all mice had lost 10-20% of weight after SE. Two pilocarpine-SE mice died suddenly overnight in their home cages at 10 and 21 days, respectively. Autopsies did not reveal causes of death and therefore sudden unexplained death in epilepsy or severe seizures are likely. Ten of these mice were randomly selected for this study. When video- electroencephalography monitored, all male CD1 mice subjected to our pilocarpine-SE model developed spontaneous recurrent seizures and/or interictal spikes (Kharatishvili *et al* 2013) similar to previous reports in the same mouse strain (Shibley and Smith 2002). Eleven control mice received methylscopalamine and pentobarbital only and 0.9% saline instead of pilocarpine. At the end of the study, the mean weights of pilocarpine-SE mice were significantly lower compared to controls, 33.92 ± 1.42 g vs. 38.60 ± 1.25 g ($p = 0.024$, $n = 10$ pilocarpine-SE, $n = 11$ control mice).

3.1.3. ^{13}C glucose administration

To be able to determine glucose metabolism, we injected animals with ^{13}C labeled glucose 15 min before microwave fixation of the head. In Papers 1 and 2, mice received a single bolus of [1-^{13}C]glucose (543 mg/kg i.p.), whereas in Paper 3 a single bolus of [1,2-^{13}C]glucose (543 mg/kg i.p.) was given. The time interval of 15 min between microwave fixation and ^{13}C glucose injection ensures substantial ^{13}C label incorporation in brain metabolites without washout, in line with previous pilot experiments using intraperitoneal injection and a study showing that ^{13}C enrichment is highest 15 min after intravenous injection of ^{13}C glucose (Hassel *et al* 1995).

3.2. Metabolic analysis

In all studies, we quantified amounts of metabolites in the cerebral cortex and HF using high-pressure liquid chromatography (HPLC) and ^{1}H NMR spectroscopy. Metabolism of ^{13}C glucose was determined using ^{1}H and ^{13}C NMR spectroscopy, except for Paper 3, in which ^{13}C glucose metabolism in the HF was evaluated using gas chromatography–mass spectrometry (GC-MS) in addition to ^{1}H NMR spectroscopy. In all studies, mice were terminated using microwave fixation. Microwave fixation is considered the most humane method to euthanize animals, reducing the time of post-mortem metabolism and keeping animal stress and suffering to an absolute minimum (AVMA 2013). After microwave fixation, mice were decapitated (trunk blood was collected in Paper 3) and cerebral cortices and HF were

dissected and stored at -80 °C till extraction. The HF included the dentate gyrus, hippocampus proper, subiculum, but not entorhinal cortex.

To obtain water-soluble molecules for subsequent in vitro analysis, tissue samples were extracted using the perchloric acid method (Paper 1 and 2) or the methanol-chloroform-water method (Paper 3). Both methods ensure high total tissue metabolite yield with low variability (Le Belle *et al* 2002). In Paper 3, concerns were raised that the salt content in samples extracted using the perchloric acid technique could decrease spectral quality for our new QCI CryoProbe™ 600MHz ultrashielded Plus magnet (Bruker BioSpin GmbH, Rheinstetten, Germany). Therefore, we decided to use the methanol-chloroform-water method instead, which does not involve precipitation of salt.

In Paper 3, L-2-Aminobutyric acid (α-ABA) (Sigma Aldrich, St Louis, MO, USA) was added as an internal standard for HPLC analysis. All metabolite concentrations were corrected for possible tissue loss during the extraction procedure using a factor derived from the known amount of α-ABA added to the tissue and the actual amount of the final sample quantified by HPLC.

3.2.1. ^{1}H- and ^{13}C NMR spectroscopy

NMR spectroscopy is a powerful method for investigating brain metabolism in vivo and ex vivo. By exploiting the magnetic moment of nuclei with net spin, NMR spectroscopy allows obtaining information about atoms or molecules in a sample of interest. Spin is a fundamental property of all elementary particles like mass and electrical charge meaning that a particle rotates around its axis. Nuclei with an odd number of protons and/or of neutrons possess net spin, and nuclei with net spin have magnetic moment. ^{1}H- and ^{13}C are two such nuclei, and in the works included in this thesis, ^{1}H- and ^{13}C NMR spectroscopy were used to study metabolism in extracts of HF and cerebral cortex of mice. ^{13}C NMR spectroscopy is an excellent tool for investigating the flux of ^{13}C labeled substrates through metabolic pathways and for distinguishing neuronal-astrocytic interactions in extracts of brain tissue (Rodrigues *et al* 2009; Sonnewald and Kondziella 2003).

When a sample is subjected to an external magnetic field (B_0), nuclei with net spin will align with or against the field. The nuclei precess about the magnetic field at a certain frequency termed Larmor frequency. The Larmor frequency is the product of the gyromagnetic ratio of the nucleus and the magnetic field strength. The nuclei that are oriented parallel to the field exists in a lower energy state than those oriented against the field, with a surplus of spins existing in the lower energy state compared to those in the higher energy state. The number of spins in the lower energy state increases with increasing B_0. If an electromagnetic pulse B_1 with a radio frequency that equals the Larmor frequency is applied to the nuclei, the nuclei will absorb the energy and transit to a higher energy state. This exchange of energy between two different systems with the same frequency is known as resonance. After the transition

the nuclei will return to the lower energy state (in a process called relaxation) and re-emit the electromagnetic radiation. This phenomenon is exploited by NMR spectroscopy. Samples are subjected to a large B_0 and a radio frequency B_1, and when B_1 is turned off, the tissue transmits an energy signal containing information about the tissue. The signal is detected by a receiver coil in the form of a current termed the free induction decay, which is converted from the time to frequency domain via the mathematical operation Fourier transformation, generating NMR spectra. After repeated pulses and relaxations, the signal is accumulated increasing the precision of the information.

The resonance frequency of a nucleus not only depends on the external magnetic field applied, but is also slightly affected by the electron density around the nucleus, which varies with the type of nuclei and the bonds in the molecule. This phenomenon, known as chemical shift, is essential for NMR spectroscopy and makes it possible to distinguish between different atoms in the same molecule and between different molecules. Importantly, chemical shift is constant if pH and temperature are controlled. The magnetic field of a nucleus is also affected by nearby nuclei with net spin. This effect is called spin-spin coupling or J-coupling and generates multiplets in the NMR spectra. Spin-spin couplings between nearby ^{13}C nuclei (homonuclear coupling) cause metabolites with two adjacent ^{13}C nuclei to be represented as doublets and not singlets in spectra. Spin-spin couplings between ^{13}C and ^{1}H nuclei (heteronuclear coupling) make ^{13}C NMR spectra hard to analyze. This is avoided by applying a small powered B_1 pulse in the frequencies of ^{1}H during ^{13}C NMR experiments, thus decoupling ^{1}H. However, by applying energy to the system the intensity of peaks in the ^{13}C NMR spectra increases. When quantifying ^{13}C metabolites this phenomenon, termed nuclear Overhauser enhancement (NOE), has to be corrected for. Since we do not allow ^{13}C nuclei to fully relax (to spare time, while still obtaining valid spectra) we also need to correct ^{13}C integrals for the lack of relaxation time. Correction factors were determined from two sets of ^{13}C NMR spectra obtained in a pilot study. In the first set, NMR spectra were acquired with a standard pulse angle, relaxation delay and acquisition time used in later experiments. In the second set, NMR spectra were acquired with a relaxation time of 20 s and ^{1}H decoupling initiated only during spectral acquisition, which avoids NOE. Next, correction factors for the differential NOE and relaxation effects experienced by the reference compound and the compounds were calculated.

The natural abundance of ^{13}C is only 1.1% of total carbon. Therefore it is of little use to apply ^{13}C NMR spectroscopy for studying endogenous metabolites. However, the low natural abundance can be exploited by administrating ^{13}C labeled substrates and using ^{13}C NMR analysis for determining metabolic pathways and trafficking of metabolites between different compartments. In the works included in this thesis we used [1-^{13}C]glucose (Papers 1 and 2) and [1,2-^{13}C]glucose (Paper 3). To interpret the ^{13}C results it is necessary to know the labeling patterns from metabolism of ^{13}C labeled glucose.

3.2.1.1. Labeling patterns from metabolism of [1-^{13}C]glucose (Figure 3A)

[1-^{13}C]glucose is commonly used for studying glucose and amino acid metabolism and distinguishing PC activity from that of PDH. Via glycolysis, [1-^{13}C]glucose is converted to [3-^{13}C]pyruvate, which can be converted to [3-^{13}C]alanine, [3-^{13}C]lactate or enter the TCA cycle via PDH as [2-^{13}C]acetyl-CoA. Metabolism of [2-^{13}C]acetyl-CoA in the TCA cycle gives rise to [4-^{13}C]α-ketoglutarate, which is a precursor for [4-^{13}C]glutamate. Thereafter, [4-^{13}C]glutamate may be converted to [4-^{13}C]glutamine by the astrocytic enzyme GS or to [2-^{13}C]GABA in GABAergic neurons. If [4-^{13}C]α-ketoglutarate is further converted in the TCA cycle, labeled oxaloacetate will be formed which can be transaminated to [2-^{13}C]aspartate or [3-^{13}C]aspartate. In astrocytes, [3-^{13}C]pyruvate can be converted to [3-^{13}C]oxaloacetate via PC, which can lead to the formation of [2-^{13}C]α-ketoglutarate and eventually [2-^{13}C]glutamate, [2-^{13}C]glutamine, [4-^{13}C]GABA, and [1-^{13}C]aspartate or [4-^{13}C]aspartate. Transamination of [3-^{13}C]oxaloacetate leads to [2-^{13}C]aspartate or [3-^{13}C]aspartate. Further cycling of labeled metabolites gives rise to different labeling patterns in amino acids.

3.2.1.2. Labeling patterns from metabolism of [1,2-^{13}C]glucose (Figure 3B)

In Paper 3, we injected mice with [1,2-^{13}C]glucose to obtain information about the PPP in addition to distinguishing metabolism of ^{13}C labeled pyruvate via PC from that of PDH activity. Via glycolysis, [1,2-^{13}C]glucose is metabolized to [2,3-^{13}C]pyruvate, which can be converted to [2,3-^{13}C]alanine, [2,3-^{13}C]lactate or enter the TCA cycle via PDH as [1,2-^{13}C]acetyl-CoA. Metabolism of [1,2-^{13}C]acetyl-CoA in the TCA cycle gives rise to [4,5-^{13}C]α-ketoglutarate, which is a precursor for [4,5-^{13}C]glutamate. Thereafter, [4,5-^{13}C]glutamate may be converted to [4,5-^{13}C]glutamine by the astrocytic enzyme GS or to [1,2-^{13}C]GABA in GABAergic neurons. In astrocytes, [2,3-^{13}C]pyruvate can be converted to [2,3-^{13}C]oxaloacetate via PC, which can lead to the formation of [2,3-^{13}C]α-ketoglutarate and eventually [2,3-^{13}C]glutamate, [2,3-^{13}C]glutamine and [3,4-^{13}C]GABA. Transamination of [2,3-^{13}C]oxaloacetate leads to [2,3-^{13}C]aspartate (not depicted in the figure). If [2,3-^{13}C]α-ketoglutarate and [4,5-^{13}C]α-ketoglutarate is further metabolized in the TCA cycle, labeled oxaloacetate will be formed which can be transaminated to [1,2-^{13}C]aspartate or [3,4-^{13}C]aspartate. Further cycling of labeled metabolites gives rise to different labeling patterns in amino acids.

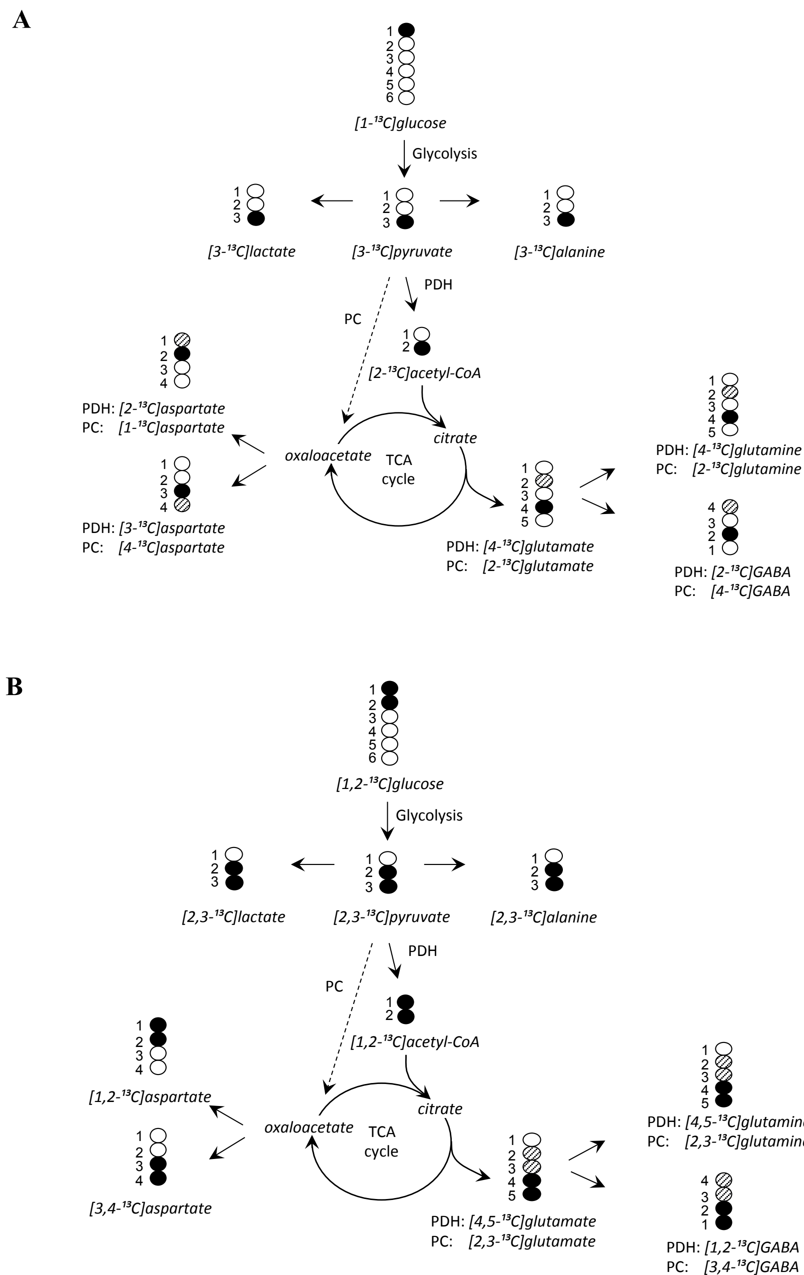

Figure 3 Simplified schematic representation of labeling patterns from **A**) [1-^{13}C]glucose and **B**) [1,2-^{13}C]glucose. Only the first turn of the TCA cycle is illustrated. Black circles indicate ^{13}C labeling. ^{13}C labeling from pyruvate carboxylase (PC) is indicated by hatched circles. Metabolism of [3-^{13}C]pyruvate via PC leads to [3-^{13}C]oxaloacetate, which can be converted to [2-^{13}C]aspartate or [3-^{13}C]aspartate (Figure 3A). Metabolism of [1,2-^{13}C]glucose leads to [1,2-^{13}C]aspartate and [3,4-^{13}C]aspartate labeled from both pyruvate dehydrogenase (PDH) and PC (Figure 3B).

3.2.1.3. Experimental setup for ^1H and ^{13}C NMR spectroscopy

In Papers 1 and 2, lyophilized samples were dissolved in 200 µL of D_2O containing 0.01% ethylene glycol as an internal standard for quantification. Samples were transferred to 5 mm Shigemi NMR microtubes (Shigemi Inc., Allison Park, PA, USA). The HF samples were analyzed using a BRUKER DRX-600 spectrometer (BRUKER Analytik GmbH, Rheinstetten, Germany), while the cortex samples were analyzed using a BRUKER DRX-500 spectrometer. The spectra were recorded at 25° C. ^1H NMR spectra were acquired on the same instruments with the following parameters: pulse angle of 90°, spectral width of 32 K data points and the number of scans was 128 and 1024 for cortical and hippocampal extracts respectively. The acquisition time was 1.36 s and relaxation delay was 10 s. Water suppression was achieved by applying a low-power pre-saturation pulse at the water frequency. Proton decoupled ^{13}C NMR spectra were obtained using a 30° pulse angle and 30 kHz spectral width with 64 K data points employing an acquisition time of 1.08 s and a relaxation delay of 0.5 s. The number of scans needed to obtain an appropriate signal to noise ratio was typically 25 000 for the cortex samples and 35 000 for the HF samples. Relevant peaks in the spectra were identified and integrated using XWINNMR software (BRUKER BioSpin GmbH, Rheinstetten, Germany).

In Paper 3, lyophilized samples were dissolved in 120 µl of D_2O (99.9%; Cambridge Isotope Laboratories) containing 0.10% ethylene glycol (Merck, Darmstad, Germany) and 0.29 g/L 2,2,3.3-d(4)-3-(Trimethylsilyl)propionic acid sodium salt (TSP) (98%; Alfa Aesar, Karlsruhe, Germany) as internal standards for quantification. Samples were transferred to SampleJet tubes (3.0x103.5 mm) for insertion into the SampleJet autosampler (Bruker BioSpin GmbH, Rheinstetten, Germany). All samples were analyzed using a QCI CryoProbe™ 600MHz ultrashielded Plus magnet (Bruker BioSpin GmbH). ^1H and ^{13}C spectra were recorded at 20 °C. ^1H NMR spectra were acquired with the following parameters: pulse angle of 90°, acquisition time of 2.66 seconds and relaxation delay of 10 seconds. The number of scans was 256. Proton decoupled ^{13}C NMR spectra were acquired with the following parameters: pulse angle of 30°, acquisition time of 1.65 seconds and a relaxation delay of 0.5 seconds, 30 kHz spectral width with 98 K data points. The number of scans needed to obtain appropriate signal to noise ratios were between 20 000 – 40 000 (adjusted for sample weight). Relevant peaks in the spectra were identified and then integrated using TopSpin™ 3.0 software (Bruker BioSpin GmbH). For a typical ^{13}C spectrum see Figure 3.

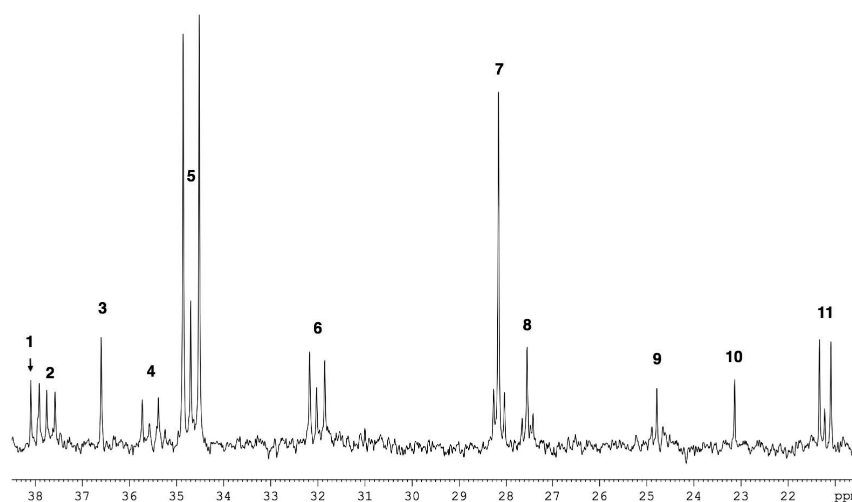

Figure 4 Typical ^{13}C NMR spectrum of a cerebral cortex extract from a mouse subjected to pilocarpine-SE and injected with [1,2-^{13}C]glucose 3.5-4 weeks later. Monolabeled metabolites give rise to singlets whereas double-labeled metabolites give rise to two peaks on either side of the singlet. It should be noted that the peaks of creatine, taurine and *N*-acetyl aspartate represent natural abundance of ^{13}C. Peak assignment: 1: [2-^{13}C]creatine; 2: [3-^{13}C]aspartate and [3,4-^{13}C]aspartate; 3: [2-^{13}C]taurine; 4: [2-^{13}C]GABA and [1,2-^{13}C]GABA; 5: [4-^{13}C]glutamate and [4,5-^{13}C]glutamate; 6: [4-^{13}C]glutamine and [4,5-^{13}C]glutamine; 7: [3-^{13}C]glutamate and [2,3-^{13}C]glutamate; 8: [3-^{13}C]glutamine and [2,3-^{13}C]glutamine; 9: [3-^{13}C]GABA and [3,4-^{13}C]GABA; 10: [6-^{13}C]*N*-acetyl aspartate; 11: [3-^{13}C]lactate and [2,3-^{13}C]lactate.

3.2.1.4. Quantification of metabolites from ^{1}H and ^{13}C NMR spectra

The total amounts and ^{13}C labeling of metabolites were quantified from the integrals of the peak areas using internal standards. In Papers 1 and 2, ethylene glycol was used as internal standard in both ^{1}H and ^{13}C spectra, whereas in Paper 3, TSP was used as internal standard in ^{1}H spectra and ethylene glycol in ^{13}C spectra. Integrals from ^{1}H spectra were corrected for number of protons constituting the peak. Integrals from ^{13}C spectra were corrected for NOE and relaxation effects relative to the internal standard, and were corrected for the 1.1% natural abundance of ^{13}C using the data obtained from ^{1}H NMR spectroscopy or HPLC. When the metabolite was double-labeled, ^{13}C natural abundance was calculated as 1.1% x 1.1% of total metabolite amount. In Paper 3, all concentrations were corrected for possible tissue loss during the extraction procedure using a factor derived from the known amount of α-ABA added to the tissue and the actual amount of the final sample quantified by HPLC.

3.2.2. HPLC

HPLC is a technique that separates and quantifies a mixture of compounds. The compounds are dissolved in a liquid mobile phase and pumped through a column containing the stationary phase. Depending on the affinity of the compounds to the column material and the mobile phase, the time to move through the stationary phase differs. A detector at the other side of the column detects the compounds at their specific retention times.

3.2.2.1. Experimental setup of HPLC

To determine the total amounts of amino acids the samples were analyzed using a Hewlett Packard 1100 System (Agilent Technologies, Santa Clara, CA, USA) with fluorescence detection, after derivatization with o-phthaldialdehyde. The components were separated with a ZORBAX SB-C18 (4.6 x 150 mm, 3.5 micron) column from Agilent using 50 mM sodium phosphate buffer (pH 5.9) with 2.5% tetrahydrofurane and methanol (98.75%) with tetrahydrofurane (1.25%) as eluents. Compounds were quantified by comparison with a standard curve derived from a standard solution of metabolites run repeatedly with 15 samples intervals.

In Papers 1 and 2, we determined the total amounts of monoamines and acid metabolites using an Agilent 1200 system (Agilent, USA) with an electrochemical detector (Coulochem III, ESA, USA). The components were separated with an Eclips XDB-C18 column (4.6x150 mm, 5 micron, Agilent) with an aqueous mobile phase (0.90 mL/min) containing 90 mM NaH2PO4, 50 mM citric acid, 0.1 mM EDTA, 0.5 mM octanesulfonic acid and 7% methanol solution. The amounts of monoamines were quantified by comparison with a standard curve derived from standard solutions of monoamines run repeatedly after every 15 samples.

3.2.3. GC-MS

GC-MS was applied in Paper 3 to analyze the percent labeling incorporated from [1,2-^{13}C]glucose into amino acids in hippocampal extracts. In analogy to HPLC, compounds are separated according to their affinity to the column material and the mobile phase, in this case helium. Next, molecules are shattered into ionized fragments and detected according to their mass to charge ratio by a mass spectrometer.

3.2.3.1. Experimental setup of GC-MS

Aliquots of the samples were dissolved in 0.05 M HCl, followed by lyophilization. Organic acids and amino acids were extracted into an organic phase of ethanol and benzene, dried under air and reconstituted in *N*,*N*-dimethylformamide (Sigma Aldrich) before derivatization with *N*-Methyl-*N*-(t-butyldimethylsilyl)trifluoroacetamide in 1% t-butyldimethylchlorosilane (both Regis Technologies Inc., Morton Grove, IL, USA). Compounds were analyzed with an Agilent 6890N gas chromatograph linked to an Agilent 5975B mass spectrometer with an electron ionization source. Results for metabolites were corrected for natural abundance of ^{13}C using standard solutions that were acquired concurrently with the samples.

3.3. Statistics

In all studies, the number of samples for each group varied between analytical methods due to experimental errors.

Paper 1: Statistical analysis was performed using the 2-tailed unpaired Student's *t*-test, and $p \leq 0.05$ was regarded as significant. Data are represented as mean ± SD.
Paper 2: Statistical analysis of metabolites was performed using an one-way ANOVA followed by a least significant difference post-hoc test, and $p < 0.05$ was regarded as significant. Data of metabolites are represented as mean ± SEM. Analysis of seizure severity and latency was performed using a Kruskal-Wallis test (nonparametric ANOVA) followed by Mann-Whitney U test. Severity and latency data are represented as mean ± SEM.
Paper 3: Statistical analysis was performed using the 2-tailed unpaired Student's *t*-test, and $p < 0.05$ was regarded as significant. Data are represented as mean ± SEM.

4. Synopsis of papers

4.1. Paper 1

Smeland O*, Meisingset T*, Borges K, Sonnewald U
Dietary supplementation with acetyl-L-carnitine improves brain energy metabolism in healthy mice and increases noradrenaline and serotonin content
*Neurochemistry International, 2012 Jul, vol 61 (1) p 100-107, * Joint first authors*

Aims: ALCAR has been tried out as a therapeutic supplement in various brain disorders for more than two decades. Until now, the effects of ALCAR on brain metabolism have mainly been studied after acute administration in rats. We aimed to perform a comprehensive analysis of glucose, energy, amino acid and monoamine neurotransmitter metabolism after continuous ALCAR supplementation in healthy mice.
Methods: Mice received normal drinking water or drinking water supplemented with ALCAR (1.5g/L) for 25 days. 15 min before microwave fixation of the head, animals were injected with [1-^{13}C]glucose. Extracts of HF and cerebral cortex were investigated using ^{1}H and ^{13}C NMR spectroscopy and HPLC.
Results: We detected increased amounts of glucose in both HF and cortex of mice supplemented with ALCAR. In the HF, [1-^{13}C]glucose content was increased. The mean value of cortical [1-^{13}C]glucose amount was increased in ALCAR mice, however the difference between the groups did not reach statistical significance ($p = 0.06$). The amount of lactate was not significantly altered by ALCAR administration, whereas the levels of [3-^{13}C]lactate were lower in both brain regions investigated. The amount of alanine was significantly decreased in the HF, but unaltered in cortex. Total amounts and ^{13}C labeling of glutamate and glutamine were unaffected by ALCAR treatment in both brain regions investigated, whereas the level of GABA was reduced in the HF. The sum of adenosine phosphate (AMP) + ADP + ATP, and the levels of phosphocreatine and *myo*-inositol were all significantly increased in the cortex of mice treated with ALCAR. ALCAR administration significantly increased the level of hippocampal noradrenaline, whereas the levels in cortex were similar compared with control. Finally, the amount of serotonin was significantly increased in the cortex of ALCAR-mice.
Conclusions: We report that chronic ALCAR supplementation in healthy mice increased energy availability and decreased glucose consumption in both HF and cortex. The findings suggest that ALCAR preferentially enters a TCA cycle compartment geared for generation of energy and not amino acid synthesis, since [1-^{13}C]glucose incorporation into amino acids was unaltered. The increase in levels of noradrenaline and serotonin are relevant to previously reported antidepressant effects of ALCAR treatment.

4.2. Paper 2

Smeland O*, Meisingset T*, Sonnewald U
Dietary supplementation with acetyl-L-carnitine in seizure treatment of pentylenetetrazole kindled mice
*Neurochemistry International, 2012 Sep, vol 61 (4) p 444-454, * Joint first authors*

Aims: We hypothesized that ALCAR supplementation could reduce seizures and normalize brain metabolism in an animal model of epilepsy. ALCAR has several properties that may suggest an antiepileptic effect, such as protection against glutamate toxicity (Forloni *et al* 1994), a favorable effect on energy homeostasis (Aureli *et al* 1990; Aureli *et al* 1998; Scafidi *et al* 2010b), and lowering oxidative damage and improving mitochondrial function (Liu *et al* 2002). Moreover, pretreatment with l-carnitine before administration of a single convulsive dose of PTZ to mice prolonged latency to seizures and reduced the frequency of clonic seizures in a dose-dependent manner (Yu *et al* 1997). We reasoned that ALCAR might mimic the anticonvulsive effects of fasting and the ketogenic diet. Since antiquity, fasting has been used to control epilepsy and fasting leads to increased production of ketone bodies that enter the brain and serve as energy substrates. The ketogenic diet mimics the metabolic state of fasting and is anticonvulsive in various forms of epilepsy (Hartman and Stafstrom 2012). Moreover, plasma levels of acylcarnitines increase during fasting in parallel with ketogenic bodies (Brass and Hoppel 1978), and the plasma level of ALCAR is increased in children on the ketogenic diet (Hack *et al* 2006). Like the ketogenic diet, ALCAR treatment might serve as an alternative energy source to brain cells with altered amino acid homeostasis and decrease seizure susceptibility. For our purpose, we wanted to test ALCAR in an animal model of epilepsy in which the ketogenic diet was proven anticonvulsive. We decided upon using the PTZ kindling model described by Hansen et al (2009).
Methods: Mice received normal drinking water or drinking water supplemented with ALCAR (1.5g/L) or valproate (3.3g/L) for 25 days. The animals were injected with PTZ (43 mg/kg) or saline three times a week for three weeks (Hansen *et al* 2009). Upon PTZ injection seizure severity and latency to clonic-tonic seizures were scored. 15 min before microwave fixation of the head, animals were injected with [1-^{13}C]glucose. Extracts of HF and cerebral cortex were investigated using ^{1}H and ^{13}C NMR spectroscopy and HPLC.
Results: ALCAR treatment did not affect kindling progression in PTZ kindled mice. Valproate, on the other hand, delayed the progression of kindling in the initial stage of the experiment evidenced by a significantly lower Racine score at injection day 3. Latency to seizures was not affected by either valproate or ALCAR. PTZ kindling caused glucose hypometabolism, evidenced by a reduction in both glycolysis and TCA cycle turnover in both brain regions investigated, affecting both glutamatergic and GABAergic neurons. The amount of glutamate was significantly decreased in the

HF of PTZ kindled mice, but not in cortex. ALCAR was able to attenuate PTZ induced reductions in lactate and [3-^{13}C]alanine and an increase in dopamine in the HF. Moreover, ALCAR normalized the levels of *myo*-inositol and succinate in the cortex.

Conclusions: ALCAR supplementation did not affect kindling progression in this PTZ kindling model in mice. However, ALCAR supplementation normalized some metabolic parameters, which may indicate beneficial effects of ALCAR on metabolism in the epileptic mouse brain.

4.3. Paper 3

Smeland O, Hadera M, McDonald T, Sonnewald U, Borges K
Brain mitochondrial metabolic dysfunction and glutamate level reduction in the pilocarpine model of temporal lobe epilepsy in mice
Journal of Cerebral Blood Flow and Metabolism, 2013, In print

Aims: The mechanisms underlying the pathogenesis of TLE still remain unclear, although increasing evidence points to a disturbance in amino acid neurotransmitter homeostasis and energy metabolism. We aimed to investigate glucose, amino acid and energy metabolism in the pilocarpine-SE model of TLE in mice.

Methods: To investigate glucose metabolism, we injected mice 3.5 – 4 weeks after SE with [1,2-^{13}C]glucose before microwave fixation of the head. Using ^{1}H and ^{13}C NMR spectroscopy, GC-MS and HPLC, we quantified metabolites and ^{13}C labeling in extracts of cerebral cortex and HF.

Results: We detected decreased hippocampal levels of glutamate, glutathione and alanine in pilocarpine-SE mice compared to controls. Moreover, the contents of NAA, succinate and NAD(P)H were decreased in HF indicating impairment of mitochondrial function. Additionally, the reduction in ^{13}C enrichment of hippocampal citrate and malate suggests decreased TCA cycle turnover in this region. In cortex, we found reduced ^{13}C labeling of glutamate, glutamine and aspartate via the PC and PDH pathways, suggesting slower turnover of these amino acids and/or the TCA cycle. ^{13}C enrichment of metabolites via the PPP was negligible in both brain areas.

Conclusions: Altogether the metabolic alterations partly resemble those reported for human TLE and rat models of chronic epilepsy, such as glutamate content reduction and mitochondrial metabolic dysfunction in the HF. The decreased turnover of important metabolites within and derived from TCA cycle intermediates in both brain regions are consistent with impaired function of the TCA cycle in mice with spontaneous recurrent seizures. Targeting these impaired mitochondrial functions might be promising for the treatment of drug-resistant epilepsy.

5. Discussion

In the studies constituting this thesis, we obtained neurochemical maps of mice supplemented with ALCAR and two mouse models of TLE. The anticonvulsive effect of ALCAR was evaluated in the PTZ kindling model and compared to that of valproate. Using ^{1}H NMR spectroscopy and HPLC, we investigated metabolism of glucose, amino acids, monoamines and energy related metabolites in extracts of cerebral cortex and HF. Additionally, ^{13}C NMR spectroscopy and GC-MS were performed to provide dynamic pictures of metabolic pathways using ^{13}C labeled glucose.

5.1. Methodological considerations

In Paper 1, the differences in metabolism between the two groups represent the effect of ALCAR supplemented in the drinking water for 25 days. The variability in water consumption within the groups was small (9.9 ± 0.6 ml per day in ALCAR-control mice and 7.8 ± 1.2 ml per day in control mice) indicating that the administration procedure was adequate. Mice received a daily ALCAR dose of about 500 mg/kg in accordance with effective regimens in the literature (Hagen *et al* 2002; Mollica *et al* 2001). There are, however, other ways to administer ALCAR to animals. An alternative would be to daily inject mice with ALCAR ensuring a more controlled design independent of water intake. The advantage of our procedure was to reduce the animal stress due to repeated injections, which is why we chose it. A third and perhaps preferential option would be to orally administer a finite dose of ALCAR to animals, which is a more labor-intensive approach, but avoids the problem of repeated injections. Moreover, we should have measured the levels of glucose and ALCAR in blood and brain tissue to help interpretation of the metabolic data.

In Paper 2, the intervention groups were injected with PTZ three times a week for three weeks, and two groups were supplemented with either ALCAR or valproate for 25 days. Importantly, all groups of mice injected with PTZ were fully kindled (behavioral stage 4-5) at the final day of the study, meaning that the metabolic data represent a time point when all intervention groups had experienced similar seizure severity. The control group received saline injections (and experienced no seizures, to our knowledge). We did not investigate metabolism in the cortex of PTZ-valproate mice but only HF, since HF is considered the region of interest in TLE and valproate was primarily included in the experiment as a positive control for seizure evaluation. In retrospect, however, it would have been interesting to include the metabolic data from this group in the analysis of cortex, particularly since cortex was similarly affected as HF in PTZ kindled mice. As described in the methods section, we utilized a similar PTZ kindling protocol as Hansen et al. (2009). We used the same mouse strain from the same breeding center, and the same PTZ dose, injection regime and seizure scoring system. Nevertheless, our PTZ kindled mice experienced considerably

higher seizure severity than those reported by Hansen et al. (2009). Moreover, Hansen et al. (2009) reported no mortality, whereas two mice died in our PTZ-control group and one in the PTZ–ALCAR group, indicating a larger PTZ effect in our mice. It is possible that the slight difference in age between the mice in the two studies contributed to the difference in seizure susceptibility; our mice were 4 weeks of age at the start of kindling compared to 5 weeks in the study of Hansen et al. (2009). Indeed, Löscher et al. (1991) pointed out that the seizure threshold after PTZ injection heavily relies on the age of animals and may vary even within one week.

In Paper 3, differences between control and pilocarpine-SE mice represent the effect of both epileptogenesis and seizure activity on brain metabolism. It is possible that inter-animal variability in the number and severity of seizures contributed to variability in metabolic data. However, seizure data was not acquired for each animal. In a study using the same mouse model, the mean seizure frequency in the chronic phase was 0.5 ± 0.3 per day and all seizures were secondarily generalized (behavioral stage 4–5 according to the Racine scale) (Kharatishvili *et al* 2013). Pilocarpine-SE mice received less [1,2-^{13}C]glucose compared to controls due to significantly lower body weights. This could lead to decreased enrichment of blood glucose with [1,2-^{13}C]glucose. However, the ^{13}C enrichment of glucose, lactate and alanine was similar in both brain regions indicating that ^{13}C enrichment of glucose in the blood was similar between the two groups.

In previous studies, our group has co-administered [1-^{13}C]glucose with [1,2-^{13}C]acetate to investigate metabolism in brain tissue using ^{13}C NMR spectroscopy (Melø *et al* 2005; Sonnewald and Kondziella 2003). Acetate is selectively taken up by astrocytes (Waniewski and Martin 1998) and co-administration of this substrate gives additional information about the astrocytic compartment. In the studies of Papers 1 and 2 we intended to analyze brain extracts using GC-MS. Since GC-MS does not allow for distinguishing the position of carbon atoms within molecules, co-administration with [1,2-^{13}C]acetate would confound the interpretation of GC-MS data and was therefore not performed. In the end, we did not analyze extracts using GC-MS, since we were satisfied with our results using NMR spectroscopy. In Paper 3, we decided to utilize [1,2-^{13}C]glucose to provide information about the PPP. Co-administration with ^{13}C labeled acetate would confound this information and was therefore not done. Unfortunately, PPP activity was below our detection limit in any metabolite in the brain regions analyzed.

5.2. Glucose metabolism

Recently, Lei et al. (2010) demonstrated that cerebral hypometabolism of glucose is marked by increases in cerebral glucose content at the entire range of plasma glucose levels. Chronic ALCAR treatment has been shown not to alter serum concentrations of glucose (Freo *et al* 2009) or the uptake of glucose into rat brain slices (Tanaka *et al* 2003). In Paper 1, we detected increased levels of glucose in both HF and cortex of

mice supplemented with ALCAR. Coupled with the reduced levels of [3-^{13}C]lactate in both brain regions, the findings suggest a glucose sparing effect of ALCAR treatment decreasing the anaerobic metabolism of glucose.

In Paper 2, the glucose content of PTZ-control mice was increased in cortex, while the increase in hippocampal glucose was not statistically significant ($p = 0.14$). In an experimental protocol similar to ours, PTZ kindled mice displayed increased blood glucose levels (Yudkoff *et al* 2003), whereas injection of a subconvulsive dose of PTZ to rats did not affect blood glucose concentrations (Eloqayli *et al* 2004). Higher blood glucose could explain the increase in cerebral glucose content in PTZ-control mice due to increased concentration gradient. However, the reduction in the levels of lactate, [3-^{13}C]lactate and [3-^{13}C]alanine in the HF, and decreased amount of [3-^{13}C]lactate in cortex point to cerebral glucose hypometabolism in PTZ-control mice. In the HF, ALCAR attenuated the decrease in lactate and [3-^{13}C]alanine induced by PTZ kindling, indicating a normalizing effect on pyruvate metabolism in this region. ALCAR did not attenuate glucose hypometabolism in cortex. Valproate did not affect hippocampal glucose metabolism in PTZ kindled mice.

We found no differences in the concentrations and ^{13}C enrichments of glucose, lactate and alanine in either brain region of pilocarpine-SE mice, in contrast to that seen in PTZ kindled mice. The level of blood glucose was unaltered in pilocarpine-SE mice. The reason that glucose metabolism seemed to be more affected in PTZ kindled mice compared to pilocarpine-SE mice may be due to metabolic differences between the models or that PTZ kindled mice were in a post-ictal state of down regulated metabolism less than 30 min after final seizure.

5.3. Amino acid and neurotransmitter metabolism

In Paper 1, the total amounts and ^{13}C enrichments of amino acids, except for GABA in the HF, were unaltered. This indicates that the same amount of total and ^{13}C labeled acetyl-CoA from metabolism of [1-^{13}C]glucose entered the TCA cycle responsible for labeling amino acids regardless of ALCAR supplementation. In contrast to our findings, Aureli et al. (1998) reported that a single bolus of ALCAR prior to [1-^{13}C]glucose injection decreased “the total amount of ^{13}C label incorporation into cerebral amino acids and TCA intermediates” in 6-months-old rats (Aureli *et al* 1998). When we did a similar calculation we did not observe a decrease. The discrepancy can be explained by a higher contribution of the acetyl moiety from ALCAR to amino acid synthesis in the acute compared to chronic treatment, or the age or the animal species might have an impact (our mice were 2 months old at the end of the study). In 21-22 day-old rats, acute administration of ^{13}C labeled ALCAR lead to ^{13}C label incorporation into amino acids derived from the TCA cycle, clearly showing that ^{13}C labeled ALCAR may provide ^{13}C labeled acetyl-CoA to the TCA cycle (Scafidi *et al* 2010a). The fact that we found unaltered ^{13}C enrichment of amino acids from metabolism of [1-^{13}C]glucose indicates that either 1) the acetyl-moiety of

ALCAR *was not* incorporated into amino acids in our study; 2) the acetyl-moiety of ALCAR *was* incorporated into amino acids replacing another acetyl-CoA source than glucose, thus maintaining ^{13}C enrichment of amino acids from metabolism of [1-^{13}C]glucose; or 3) the acetyl-moiety of ALCAR *was* incorporated into amino acids replacing acetyl-CoA from glucose, but the difference in ^{13}C enrichment of amino acids from metabolism of [1-^{13}C]glucose was not sufficient to generate a statistically significant result. A combination of the latter two explanations is probably correct, as it is unlikely that acetyl-CoA from ALCAR was not incorporated into amino acids in our study.

In Paper 2, we demonstrated that PTZ kindling substantially affected amino acid metabolism in both brain regions of mice. The reductions in percent ^{13}C enrichment and amount of [4-^{13}C]glutamate, [2-^{13}C]GABA, [4-^{13}C]glutamine and [2-^{13}C]aspartate in the cortex of PTZ-control mice suggest that the TCA cycle turnover was decreased 30 min after the final injection of PTZ. This is supported by a decrease in the cortical level of the TCA cycle intermediate succinate. In the HF, the amounts of [4-^{13}C]glutamate and [2-^{13}C]GABA were decreased together with a decrease in the amount of glutamate. Altogether, the results indicate decreased use of glucose for the synthesis of amino acids in both glutamatergic and GABAergic neurons in the early post-ictal phase. Interestingly, ALCAR administration seems to specifically affect GABA metabolism. In Paper 1, we showed that ALCAR supplementation decreased the concentration of GABA in the HF of normal mice (which do not intuitively suggest an antiepileptic potential of ALCAR). In Paper 2, ALCAR supplementation decreased the ^{13}C enrichment from [1-^{13}C]glucose into GABA in the cortex, indicating use of acetyl-CoA from ALCAR for GABA synthesis. This is in compliance with the study of Scafidi et al. (2010a) in which it was demonstrated higher ^{13}C enrichment in GABA than in glutamate in the mouse forebrain after [2-^{13}C]ALCAR administration, suggesting preferential metabolism of ALCAR in GABAergic neurons. Additionally, intraperitoneal administration of ALCAR for 5 days increased the content of GABA in substantia nigra of young adult mice (Fariello *et al* 1988).

Pilocarpine-SE mice displayed a similar amino acid profile as PTZ kindled mice in both brain regions. In the HF, the content of glutamate was decreased, while the contents of GABA, glutamine and aspartate remained unchanged. In cortex, there were no detectable changes in these amino acids. In cortex of pilocarpine-SE mice, the level of ^{13}C-labeling in glutamate and glutamine from both PC and PDH pathways was decreased. Furthermore, the sum of [1,2-^{13}C]aspartate + [3,4-^{13}C]aspartate, labeled both from PC and PDH, was decreased too. Reduced ^{13}C incorporation reflects either reduced use of glucose for amino acid synthesis or increased degradation of ^{13}C labeled amino acids. Since the total amounts of glutamate, glutamine and aspartate were not decreased, it follows that decreased amino acid synthesis is probably the cause, accompanied by decreased degradation. In sum, the findings in cortex indicate reduced turnover of glutamate, glutamine and aspartate, which was not evident in the GABAergic compartment. In the HF of pilocarpine-SE mice, however, the ^{13}C enrichment of GABA and aspartate was decreased, whereas

that of glutamate and glutamine remained unchanged. Furthermore, GC-MS revealed a decrease in ^{13}C enrichment of the TCA cycle intermediates citrate and malate suggesting decreased TCA cycle turnover in this region.

5.4. Metabolic dysfunction in TLE

One of the main aims of this thesis was to investigate metabolic alterations in epileptic tissue to improve our understanding of the mechanisms underlying epilepsy. These alterations may serve as biomarkers or new targets for antiepileptic treatment.

Clearly, the metabolic state in epilepsy cannot be explained by a crude imbalance between glutamate and GABA. In both pilocarpine-SE and PTZ kindled mice, we found decreased concentrations of hippocampal glutamate, in line with studies of rat models of TLE (Alvestad *et al* 2008; Melø *et al* 2005) and investigations of epileptogenic hippocampi from patients with TLE (Petroff *et al* 2002b). The concentration of hippocampal GABA, however, remained unchanged in all the above-mentioned studies, suggesting that glutamatergic metabolism is more affected than GABAergic metabolism in TLE. It is important to note that these measurements do not provide information about the dynamic balance of synaptic neurotransmitter content, but only whole tissue content, and cannot address the issue of increased extracellular glutamate concentration that is demonstrated prior to and during seizures (Cavus et al 2005; During and Spencer 1993; Kanamori and Ross 2011; Wilson et al 1996). Both the PTZ kindling model and SE models of TLE display neuronal death, especially in the HF (Franke and Kittner 2001; Sarkisian 2001). However, glutamate reduction does not appear to correlate with neuronal death in human TLE (Petroff *et al* 2002b). Thus glutamate reduction apparently results from metabolic dysfunction due altered substrate transport, decreased activity of the TCA cycle and/or various mitochondrial enzymes rather than cell death of glutamatergic neurons.

Melø et al. (2005) reported that metabolism in astrocytes was normal in the lithium-pilocarpine rat model since labeling from the astrocyte specific substrate [1,2-^{13}C]acetate was similar in control and epileptic rats. In neither PTZ kindled mice nor pilocarpine-SE mice, we could detect any difference in the total glutamine content in the HF despite the reduction in glutamate, in agreement with studies of rat models of TLE (Alvestad *et al* 2008; Melø *et al* 2005). These results seem to contrast the finding that the expression of GS was decreased in the epileptogenic HF of human TLE (Eid et al 2004; van der Hel et al 2005), indicating decreased conversion of glutamate to glutamine. However, a recent study of pilocarpine-SE rats revealed no quantitative changes in the number and volume of astrocytes expressing GS but there was a redistribution of the enzyme from distal to proximal astrocyte processes within the hippocampus (Papageorgiou et al 2011). Moreover, Melø et al. (2005) reported no differences in pyruvate carboxylation in lithium-pilocarpine rats. We, on the other hand, showed that ^{13}C-labeling in glutamate and glutamine from both PC and PDH pathways was decreased in the cortex of pilocarpine-SE mice. These findings indicate

that the decrease in glucose consumption for synthesizing glutamate and glutamine affected both the neuronal and astrocytic compartment. Moreover, we detected increased content of *myo*-inositol in the cortex of both pilocarpine-SE and PTZ kindled mice. In epilepsy, an increase of *myo*-inositol is generally considered an indication of gliosis. However, we did not detect any change in *myo*-inositol in the HF of epileptic mice, although hippocampal gliosis is a characteristic feature of TLE. Since *myo*-inositol is an important osmolyte in the brain that is particularly enriched in astrocytes (Brand *et al* 1993; Fisher *et al* 2002), the findings suggest altered water homeostasis or astrocytic regulation of *myo*-inositol in the cortex of epileptic mice that was not evident in the HF.

A characteristic feature of TLE is interictal glucose hypometabolism in the hippocampal region, which is routinely detected during presurgical evaluation of the epileptic brain using imaging methods such as positron emission tomography (O'Brien *et al* 2008). Similar to findings in the kainic acid rat model (Alvestad *et al* 2008), the pilocarpine-SE mice in Paper 3 displayed no differences in the contents of glucose, lactate or alanine, except a decreased amount of alanine in the HF, indicating normal glucose consumption. However, in both studies the incorporation of ^{13}C labeled glucose into amino acids was decreased indicating reduced formation of acetyl-CoA from glucose used for amino acid synthesis. In PTZ kindled mice and two lithium-pilocarpine rat models of TLE (Dube *et al* 2001; Melø *et al* 2005), glucose hypometabolism was evident in both hippocampal and cortical regions. Since neuronal loss does not appear to correlate with the reduction in glucose consumption (Dube *et al* 2001; O'Brien *et al* 1997), interictal hypometabolism may result from dysfunctional mitochondrial oxidative and/or glycolytic energy metabolism in epileptic tissue. Indeed, as described in the introduction, several lines of evidence point to mitochondrial dysfunction as part of the metabolic picture in TLE, which our investigations in epileptic mice seem to confirm. Similar to the content of glutamate, that of NAA was decreased in the HF of pilocarpine-SE mice, in agreement with studies of humans with TLE and rat models of TLE (Alvestad *et al* 2008; Cendes *et al* 1994; Melø *et al* 2005). Similar to glucose hypometabolism, the reduction in NAA does not correlate well with the extent of cell death in the epileptogenic hippocampus (Cohen-Gadol *et al* 2004; Kuzniecky *et al* 2001; Petroff *et al* 2002b; Vielhaber *et al* 2008). Thus it follows that NAA loss should be viewed upon as a marker of neuronal metabolic dysfunction in epileptic tissue. In PTZ kindled mice, the content of hippocampal NAA was not significantly decreased ($p = 0.23$). Furthermore, we detected decreased ^{13}C enrichment of the TCA cycle intermediates citrate and malate in the HF of pilocarpine-SE mice, suggesting decreased TCA cycle turnover in this region. Additionally, we found decreased hippocampal contents of the reducing nucleotides NAD(P)H and the TCA cycle intermediate succinate in pilocarpine-SE mice indicating impairment of mitochondrial function. These findings are in line with studies of hippocampal slices from patients with TLE demonstrating dysfunctional oxidative and/or glycolytic energy metabolism (Kann et al 2005). In PTZ kindled mice, the contents of succinate were unaltered in the HF but decreased in the cortex. We detected no significant differences in high-energy metabolites such as

phosphocreatine and ATP in either PTZ kindled mice or pilocarpine-SE mice, although reduction of these metabolites has been reported in human TLE (Pan et al 2005). In conclusion, we showed that alterations in pilocarpine-SE mice primarily concerned hippocampal metabolites related to mitochondria and the TCA cycle, whereas both brain regions were similarly affected in PTZ kindled mice and glucose metabolism was particularly altered. Thus, the results may suggest that the pilocarpine-SE model is a better model of human TLE than the mouse PTZ kindling model.

5.5. ALCAR as a therapeutic agent

To our disappointment, ALCAR supplementation did not affect kindling progression or latency to seizures in PTZ kindled mice. Valproate, on the other hand, slightly delayed the progression of kindling in the initial phase, although the occurrence of high severity seizures was not avoided in agreement with the literature (Ohno *et al* 2010). The fact that valproate did show an anticonvulsive effect demonstrates that the seizure model was valid and a potential anticonvulsive effect of ALCAR could have been evident. Despite the lack of anticonvulsive effects, ALCAR treatment produced some noteworthy metabolic alterations in PTZ kindled mice such as attenuating glucose hypometabolism in the HF. In the cortex, ALCAR did not affect glucose metabolism but normalized the level of succinate, which may indicate an improvement of mitochondrial metabolism. ALCAR treatment produced no effects on high-energy metabolites as it did in healthy mice. Interestingly, ALCAR treatment increased the content of cortical *myo*-inositol in healthy mice, whereas in PTZ kindled mice ALCAR attenuated the increase in cortical *myo*-inositol. These findings suggest that ALCAR has an astrocyte specific effect or a modulatory effect on water homeostasis. Several studies have shown that dopamine exerts a neuroprotective effect in epilepsy (Bozzi *et al* 2000; Starr 1996). In PTZ kindled mice, we showed that ALCAR attenuated the increase in hippocampal dopamine. Whether this finding indicates that ALCAR deprived the epileptic tissue of a protective measure or if ALCAR prevented the underlying cause for dopamine increase remains unanswered. With regard to GABA, ALCAR did not affect the content of this neurotransmitter in either brain region of PTZ kindled mice, although ALCAR decreased hippocampal GABA content in healthy mice. Similar to ALCAR, valproate slightly affected metabolism in PTZ kindled mice. Valproate has been shown to increase the content of GABA in several brain regions of rodents (Loscher 2002), however, we could not detect any effect of valproate on GABAergic metabolism in the HF of PTZ kindled mice. Valproate significantly increased the content of hippocampal glutathione indicating improved capacity for cytosolic oxidative reduction in this region. Although ALCAR treatment did not produce any anticonvulsive effects in the PTZ kindling model in mice, a role of ALCAR in the treatment of epilepsy cannot be excluded solely based on this study. ALCAR may be effective in other animal models

of epilepsy or in human epilepsies. Nonetheless, the clinical relevance of the metabolic alterations produced by ALCAR in epilepsy remains uncertain.

The fact that ALCAR treatment increases brain energy availability is well established in the literature and was reconfirmed in Paper 1. Overall, ALCAR supplementation seemed to improve the capacity of the cerebral cortex to buffer and provide energy, as evidenced by increased levels of phosphocreatine and the sum of AMP+ADP+ATP. The fact that the sum of creatine+phosphocreatine was not increased indicates that the increase in phosphocreatine reflects a relatively larger reservoir of high-energy phosphates, rather than an increased storage capacity for high-energy phosphates provided by creatine. This interpretation is supported by the twofold increase in the ratio of phosphocreatine/creatine in the cortex of ALCAR mice. Furthermore, the increased amount of the sum of AMP+ADP+ATP reflects increased availability of adenosine phosphates. In humans, plasma levels of adenosine and ATP increased after ALCAR administration, and the rise in adenosine levels preceded that of ATP suggesting an adenosine specific effect by ALCAR (Capecchi *et al* 1997). In the HF of mice supplemented with ALCAR, alterations in energy-related metabolites were not significantly increased. Altogether, the findings imply that the acetyl-moiety of ALCAR is used to produce energy, making the brain less dependent on glucose as energy substrate. Since ^{13}C enrichment of amino acids from [1-^{13}C]glucose was unaltered by ALCAR supplementation, the findings suggest that acetyl-CoA from ALCAR preferentially enters a TCA cycle compartment geared for energy production rather than a compartment producing TCA cycle intermediates for the synthesis of amino acids, i.e., it appears to be compartmentation of acetyl-CoA metabolism in the brain.

In line with our findings, ALCAR has already been shown to be neuroprotective in cerebral conditions with disturbed energy metabolism such as ischemia (Al-Majed et al 2006) and traumatic brain injury (Scafidi *et al* 2010b). Another brain condition in which energy metabolism may be dysfunctional is migraine (Schulz *et al* 2007), and due to the effects of ALCAR on cortical high-energy metabolites a trial of ALCAR treatment to patients with migraine has been scheduled to begin the fall of 2013 administered by the Norwegian University of Science and Technology here in Trondheim. Moreover, we demonstrated that ALCAR increased the concentrations of the monoamines noradrenaline in the HF and serotonin in the cortex of healthy mice. These findings are particularly interesting in relation to the potential role of ALCAR in the treatment of depression. Current antidepressive drugs are thought to work by increasing the synaptic content of serotonin and noradrenaline, and ALCAR was beneficial in several small trials of patients with depressive disorders and related conditions (Martinotti *et al* 2011; Pettegrew *et al* 2002; Soczynska *et al* 2008; Zanardi and Smeraldi 2006). Similar to the present state of antiepileptic treatment, the treatment of depression suffers from ineffective drugs with unpleasant side effects and novel approaches are needed. Approximately 40% of depressive patients are considered pharmacoresistant and all current antidepressants require at least 2–3 weeks to significantly improve mood (Perroud 2011). Recently, it was demonstrated that the antidepressant efficacy of ALCAR in two rat models of depression was

associated with epigenetic alterations of the metabotropic glutamate receptor 2 (Nasca *et al* 2013). Importantly, the effects were rapid and long lasting. Thus, ALCAR may be a good candidate for antidepressive treatment and more comprehensive investigations in humans are warranted (Flight 2013).

6. Concluding remarks

Although findings in mouse studies are not necessarily translational to humans, they may be highly useful in elucidating basic mechanisms of interventions or disease processes that would otherwise be unethical or impractical to extract from human studies. Hence, the work presented in this thesis aimed to contribute to the understanding of the effect of ALCAR on brain metabolism, extend our knowledge on metabolic alterations in TLE, and evaluate the anticonvulsive potential of ALCAR treatment.

In papers 2 and 3, we demonstrated that the metabolic alterations in epileptic mice partly resemble those reported in human TLE and rat models of chronic epilepsy, such as glutamate level reduction and mitochondrial metabolic dysfunction in the HF. Moreover, we revealed that the turnover of important metabolites within and derived from the TCA cycle, such as glutamate and GABA, appeared to be decreased in both brain regions. These findings are consistent with impaired function of the TCA cycle. While seizure activity fundamentally results from dysfunctional neurotransmission, metabolic alterations may contribute to and result from seizure activity. By addressing these issues, we may uncover new targets for antiepileptic treatment and improve diagnostics of epilepsy by establishing new biomarkers.

In agreement with the literature, we showed that ALCAR supplementation increased the availability of energy in the cerebral cortex of mice. Furthermore, we propose that ALCAR decreases the consumption of glucose particularly by replacing glucose as an energy fuel, since ALCAR did not seem to affect glucose incorporation into amino acids. ALCAR did not prove to be an efficient anticonvulsive agent in the PTZ kindling model and the role of ALCAR in the treatment of epilepsy remains uncertain. However, the metabolic effects of ALCAR on the healthy mouse brain, such as increasing the concentrations of noradrenaline and serotonin, show promise that ALCAR may be used in the treatment of other brain disorders including depression. It will be highly interesting to follow the research on ALCAR in the coming years.

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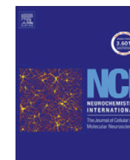

Chronic acetyl-L-carnitine alters brain energy metabolism and increases noradrenaline and serotonin content in healthy mice

Olav B. Smeland [a,1], Tore W. Meisingset [a,1], Karin Borges [b], Ursula Sonnewald [a,*]

[a] Dept. of Neuroscience, Faculty of Medicine, Norwegian University of Science and Technology, Trondheim, Norway
[b] Dept. of Pharmacology, School of Biomedical Sciences, The University of Queensland, St. Lucia QLD 4072, Australia

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A B S T R A C T

Acetyl-L-carnitine (ALCAR), the short-chain ester of carnitine, is a common dietary supplement readily available in health food stores, claimed to improve energy levels and muscle strength. ALCAR has numerous effects on brain and muscle metabolism, protects against neurotoxic insults and may be an effective treatment for certain forms of depression. However, little is known about the effect of chronic ALCAR supplementation on the brain metabolism of healthy mice. Here, we investigated ALCAR's effect on cerebral energy and neurotransmitter metabolism after supplementing the drinking water of mice with ALCAR for 25 days, providing a daily dose of about 0.5 g/kg. Thereafter the animals were injected with [1-^{13}C]glucose, and ^{13}C incorporation into and levels of various metabolites were quantified in extracts of the hippocampal formation (HF) and cortex using ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy and high performance liquid chromatography (HPLC). Increased glucose levels were detected in both regions together with a decreased amount of [3-^{13}C]lactate, but no alterations in incorporation of ^{13}C derived from [1-^{13}C]glucose into the amino acids glutamate, GABA and glutamine. These findings are consistent with decreased metabolism of glucose to lactate but not via the TCA cycle. Higher amounts of the sum of adenosine nucleotides, phosphocreatine and the phosphocreatine/creatine ratio found in the cortex of ALCAR-treated mice are indicative of increased energy levels. Furthermore, ALCAR supplementation increased the levels of the neurotransmitters noradrenaline in the HF and serotonin in cortex, consistent with ALCAR's potential efficacy for depressive symptoms. Other ALCAR-induced changes observed included reduced amounts of GABA in the HF and increased *myo*-inositol. In conclusion, chronic ALCAR supplementation decreased glucose metabolism to lactate, resulted in increased energy metabolite and altered monoamine neurotransmitter levels in the mouse brain.

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1. Introduction

Acetyl-L-carnitine (ALCAR), the short-chain ester of carnitine, is endogenously produced within mitochondria and peroxisomes and is involved in the transport of acetyl-moieties across the membranes of these organelles. Therefore, ALCAR can affect lipid, carbohydrate and amino acid, as well as energy metabolism. Since the early 1990s, much attention has been directed towards the possible role of ALCAR as a therapeutic agent in aging, disorders of the brain and its mechanism of action (reviewed by Jones et al. (2010)). Plasma and CSF concentrations of ALCAR increase after oral administration, and the compound is transported across the blood brain barrier by the organic cation/carnitine transporter OCTN2 (Kido et al., 2001; Parnetti et al., 1992). Carnitine transporters from the OCTN family are present on both neurons and astrocytes (Januszewicz et al., 2010, 2009). In rat brain cells, the acetyl moiety of ALCAR may be used for the biosynthesis of acetylcholine (Dolezal and Tucek, 1981), fatty acids (Ricciolini et al., 1998), and amino acids (Scafidi et al., 2010). Acute ALCAR administration altered rat brain energy homeostasis by increasing phosphocreatine and decreasing lactate and inorganic phosphate levels (Aureli et al., 1990), stimulating glycogen synthesis (Aureli et al., 1998), and regionally increasing [^{14}C]2-deoxyglucose labeling measured by autoradiography in certain brain regions (Ori et al., 2002). Also, chronic ALCAR increased [^{14}C]2-deoxyglucose labeling in similar regions of the rat brain (Freo et al., 2009), as well as the

Abbreviations: ALCAR, acetyl-L-carnitine; HF, hippocampal formation; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; GABA, γ-aminobutyric acid; 5-HIAA, 5-hydroxyindoleacetic; 5-HT, serotonin; HVA, homovanillic acid; MDMA, 3,4-methylenedioxymethamphetamine; NA, noradrenaline; NAA, N-acetylaspartate; NMR, nuclear magnetic resonance; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid.

* Corresponding author. Address: Dept. Neuroscience, Faculty of Medicine, Olav Kyrresgt. 9, N-7030 Trondheim, Norway. Tel.: +47 73 590492; fax: +47 73 598655.
E-mail addresses: olavbjer@stud.ntnu.no (O.B. Smeland), torewerg@stud.ntnu.no (T.W. Meisingset), k.borges@uq.edu.au (K. Borges), ursula.sonnewald@ntnu.no (U. Sonnewald).
[1] Joint first authors.

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enzyme activities of the sodium potassium and the calcium magnesium ATP-ases by about 30% (Villa et al., 2011).

ALCAR has both antioxidant and anti-apoptotic properties (Ishii et al., 2000; Liu et al., 1993) and can protect against various neurotoxic insults such as excessive glutamate (Forloni et al., 1994) and amyloid-ß exposure (Virmani et al., 2001). Treatment with ALCAR improved neurological outcome and energy metabolism in various animal models of ischemia (Aureli et al., 1994; Rosenthal et al., 1992) and aging (reviewed by Ames and Liu (2004), Jones et al. (2010)). In light of such promising findings in animal models, ALCAR has been tested in several clinical trials for various disorders. Small beneficial clinical effects have been reported for Alzheimer's disease in a meta-analysis of double blind randomized controlled clinical trials (Montgomery et al., 2003). However, a Cochrane review found no convincing effects (Hudson and Tabet, 2003). There is some evidence from small clinical trials with 24–67 patients that ALCAR can decrease symptoms of depression in the elderly (Garzya et al., 1990; Tempesta et al., 1987), fibromyalgia patients (Rossini et al., 2007) and patients with minimal hepatic encephalopathy (Malaguarnera et al., 2011).

Most psychiatric treatment is centered on altering monoamine neurotransmitter homeostasis. There are few reports on the effect of ALCAR on monoamine metabolism in the brain and they are restricted to rats: acute ALCAR pretreatment before 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) injection prevented mitochondrial damage and also loss of serotonin (5-HT) (Alves et al., 2009). In the control group acute ALCAR only increased the level of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA), but not 5-HT, in one of six investigated brain regions, namely the prefrontal cortex. In a model of attention deficit hyperactive disorder, chronic ALCAR treatment restored the noradrenaline (NA) level and the serotonin turnover ratio (5-HIAA/5-HT) in specific brain regions towards the values of normal young rats. In normal young rats the only change was an increased serotonin turnover ratio in the cingulate cortex (Adriani et al., 2004).

In summary, there is a lack of studies on the effect of chronic ALCAR administration on brain metabolism in healthy non-elderly animals other than rats. Specifically, this is the case for monoamine neurotransmitters; information undoubtedly important for understanding, evaluating and exploiting chronic ALCAR as a therapeutic agent. Here, we give a comprehensive metabolomic analysis which includes; glucose, energy, amino acid and monoamine neurotransmitter metabolism after chronic ALCAR supplementation in mice, showing for the first time increases of noradrenaline and serotonin levels in the healthy brain.

2. Materials and methods

2.1. Chemicals

[1-^{13}C]glucose (99% ^{13}C) and D_2O (99.9%) were purchased from Cambridge Isotope Laboratories (Woburn, MA, USA), ethylene glycol from Merck (Darmstadt, Germany), and acetyl-L-carnitine from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of the purest grade available from local commercial sources.

2.2. Animals

The Norwegian National Animal Research Authority and the local ethics committee approved the experimental procedures. Fourteen male four week old NMRI mice with an average weight of 25 ± 0.7 g (*n* = 14; Taconic, Ejby, Denmark) were used in the experiment. Nine mice were used in the intervention group and five as controls. All animals were maintained under standard laboratory conditions on a 12/12 h light/dark cycle (lights on at 7AM), at a constant temperature of 22 °C and a humidity of 60%. Animals were housed in individual cages with free access to food and water. The mice were acclimatized to the above conditions for 1 week before the start of the experiments.

In accordance with previous studies, mice received drinking water containing ALCAR (1.5 g/L, pH adjusted to 6) ad libitum (Hagen et al., 2002; Mollica et al., 2001) drinking an average of 9.9 ± 0.6 ml per day (calculated by weighing the water bottles), which provides a daily ALCAR dose of 496 ± 21 mg/kg body weight. Control mice receiving filtered tap water drank 7.8 ± 1.2 ml per day. There was no difference in the weight gain between the groups. Both groups received saline injections (10 ml/kg i.p.) three times a week until the last day, due to the fact that they were part of a bigger study examining the kindling epilepsy model (data not shown). After 25 days, mice were injected with 543 mg/kg [1-^{13}C]glucose (i.p., using a 0.3 M solution) and 15 min later were subjected to microwave fixation of the head at 4 kW for 1.7 s (Model GA5013, Gerling Applied Engineering, Modesto, CA, USA), a time point that ensures substantial label incorporation without washout (Hassel et al., 1995). The brains were removed and the cortices and the HFs were dissected and stored at −80 °C till extraction. The tissue samples were homogenized in 0.7% perchloric acid using a Vibra Cell sonicator (Model VCX 750, Sonics & Materials, Newtown, CT, USA), potassium perchlorate precipitated by neutralization with 1 M KOH, and supernatants containing metabolites lyophilized.

2.3. High performance liquid chromatography (HPLC)

To determine the total amount of glutathione, the samples were analyzed using a Hewlett Packard 1100 System (Agilent Technologies, Palo Alto, CA, USA) with fluorescence detection, after derivatization with o-phtaldialdehyde and a standard curve derived from a standard solution of glutathione (Geddes and Wood, 1984). The components were separated with a ZORBAX SB-C18 column (4.6 × 150 mm, 3.55 micron, Agilent) using 50 mM sodium phosphate buffer (pH 5.9) with 2.5% tetrahydrofurane and methanol/tetrahydrofurane (98.75%/1.25%) as eluent.

To quantitate the levels of monoamines NA, 5-HT and dopamine (DA) and acid metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), an Agilent 1200 system with an electrochemical detector (Coulochem III, ESA, Sunnyvale, CA, USA) was used. Components were separated with an Eclipse XDB-C18 column (4.6 × 150 mm, 5 micron, Agilent) with an aqueous mobile phase (0.90 mL/min) containing 90 mM NaH_2PO_4, 50 mM citric acid, 0.1 mM EDTA, 0.5 mM octanesulfonic acid and 7% methanol. A standard curve derived from standard solutions of monoamines was run repeatedly at 15 samples intervals for quantitation.

2.4. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy

Lyophilized samples were dissolved in 200 μL D_2O containing 0.1% ethylene glycol as an internal standard for quantitation and re-adjusted to pH 6.5–7.5 Spectra were recorded at 25 °C using a BRUKER DRX-600 for HF and a BRUKER DRX-500 spectrometer for cortex samples (both BRUKER Analytik GmbH, Rheinstetten, Germany). ^{1}H-NMR spectra were acquired on the same instruments with the following parameters: pulse angle of 90°, spectral width of 32 K data points and the number of scans was 128 and 1024 for cortical and HF extracts respectively. Acquisition time was 1.36 s and relaxation delay 10 s. Water suppression was achieved by applying a low-power pre-saturation pulse at the water frequency. Proton decoupled ^{13}C-NMR spectra were obtained using a 30° pulse angle and 30 kHz spectral width with 64 K data points employing an acquisition time of 1.08 s and a

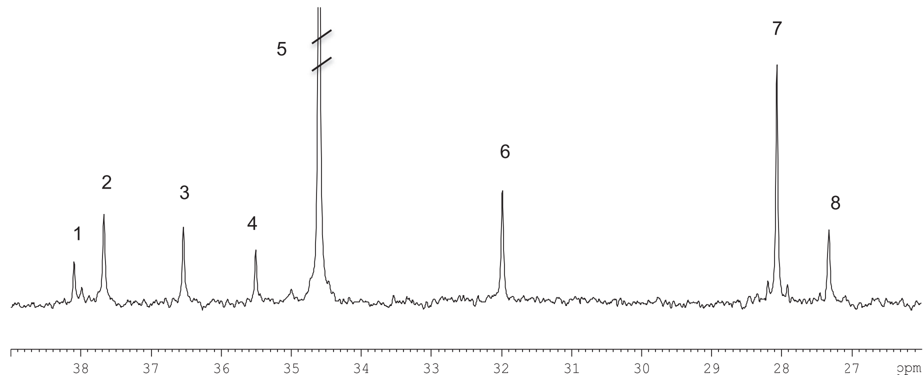

Fig. 1. Typical ^{13}C-NMR-spectrum of a tissue extract of cerebral cortex from a ALCAR-treated mouse. Peak assignment: 1: creatine C-3; 2: aspartate C-3; 3: taurine C-3; 4: GABA C-2; 5: glutamate C-4 (peak is truncated); 6: glutamine C-4; 7: glutamate C-3; 8: glutamine C-3.

relaxation delay of 0.5 s. The number of scans needed to obtain an appropriate signal to noise ratio was typically 25,000 for cortex and 35,000 for HF samples.

Relevant peaks in the spectra were identified and integrated using XWIN NMR software (BRUKER BioSpin GmbH, Rheinstetten, Germany). The total amounts and ^{13}C labeling of metabolites were quantified from the areas under the peaks using ethylene glycol as an internal standard. Correction for natural abundance, nuclear Overhauser enhancement and relaxation effects relative to the internal standard were applied to all relevant resonances. For a typical ^{13}C-NMR spectrum see Fig. 1.

2.5. Labeling patterns from metabolism of [1-^{13}C]glucose

The metabolism of [1-^{13}C]glucose is described in Fig. 2. Via glycolysis, [1-^{13}C]glucose is converted to [3-^{13}C]pyruvate, which can be further converted to [3-^{13}C]alanine, [3-^{13}C]lactate or [2-^{13}C]acetyl-CoA. [2-^{13}C]acetyl-CoA may enter the TCA cycle through condensation with oxaloacetate to form citrate. Eventually the TCA cycle intermediate α-[4-^{13}C]ketoglutarate is formed, which is the precursor of [4-^{13}C]glutamate. Thereafter, [4-^{13}C]glutamate may be transformed to [4-^{13}C]glutamine in astrocytes due to the astrocyte specific localization of glutamine synthetase (Norenberg and Martinez-Hernandez, 1979) and to [2-^{13}C]GABA in GABAergic neurons. If α-[4-^{13}C]ketoglutarate is further converted in the TCA cycle it gives rise to different labeling patterns in these amino acids (Alvestad et al., 2008). In astrocytes [3-^{13}C]pyruvate can be converted to [3-^{13}C]oxaloacetate via pyruvate carboxylase (Patel, 1974) which can lead to the formation of [2-^{13}C]glutamate, [2-^{13}C]glutamine and [4-^{13}C]GABA.

2.6. Data analysis

Statistics were performed using the 2-tailed unpaired Student's t-test, and $p \leqslant 0.05$ was regarded as significant. Data are presented as mean ± SD. Due to technical problems, the number of analyses varied between analytical methods.

3. Results

3.1. Glucose-derived and related metabolites

Injection of [1-^{13}C]glucose led to labeling of many metabolites as shown in a typical ^{13}C-NMR spectrum from cerebral cortex

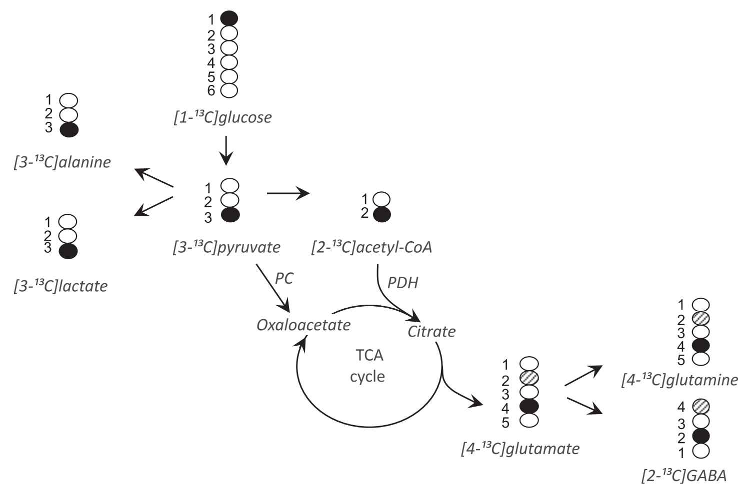

Fig. 2. Schematic presentation of isotopomers derived from [1-^{13}C]glucose via [2-^{13}C]acetyl-CoA. Black circles indicate ^{13}C labeling from pyruvate dehydrogenase (PDH) and hatched circle ^{13}C labeling from pyruvate carboxylase (PC). Only the first turn of the TCA cycle is illustrated.

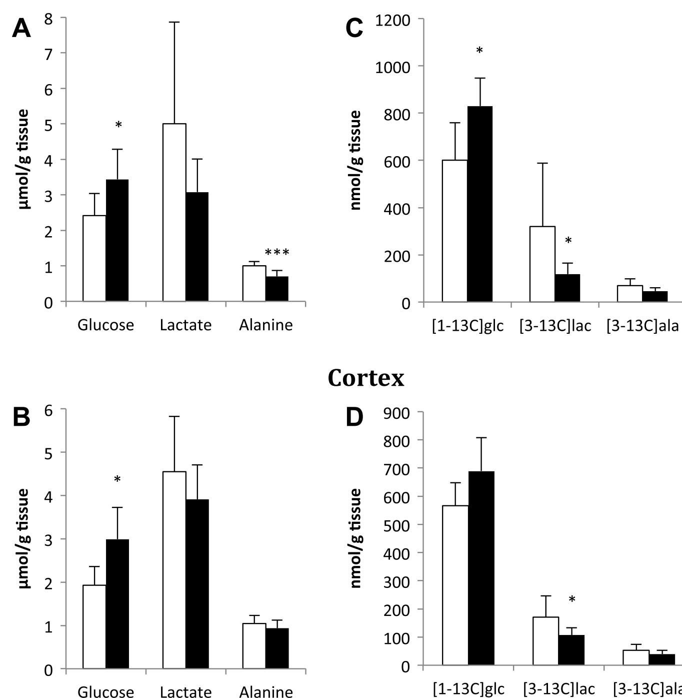

Fig. 3. Glucose metabolism in brain extracts of hippocampal formation and cortex from control (white bars) and ALCAR-treated mice (black bars). Levels of glucose, lactate and alanine quantified by ^{1}H-NMR spectroscopy (A and B) and [1-^{13}C]glucose, [3-^{13}C]lactate and [3-^{13}C]alanine obtained by ^{13}C-NMR spectroscopy are shown (C and D). Data represent mean ± SD of four control mice and nine ALCAR-treated mice for hippocampal formation, and five control mice and nine ALCAR-treated mice for cortex. $^{*}p \leqslant 0.05$, $^{***} \leqslant 0.005$, statistically significantly different from control group, analyzed by Student's *t*-test.

extract of an ALCAR-treated mouse (Fig. 1). Only the part of the spectrum containing the C-4 and C-3 glutamate and glutamine, C-2 aspartate and C-2 and C-3 GABA peaks is shown. The labeling patterns of metabolites labeled from [1-^{13}C]glucose via [2-^{13}C]acetyl-CoA from the first turn of the TCA cycle are depicted in Fig. 2. The C-2 and C-3 positions are labeled in the second and subsequent turns of the TCA cycle (not shown).

The total and labeled amounts of glucose, lactate, alanine and amino acids were quantified using ^{1}H- and ^{13}C-NMR spectroscopy. In the HF the total amounts of glucose and alanine were increased by 43% ($p = 0.05$) and decreased by 31% ($p = 0.004$), respectively after 25 days of ALCAR treatment (Fig. 3). Furthermore, lactate content in the ALCAR treated group compared to control was decreased by 38% ($p = 0.08$). In the cortex the glucose content was increased by 55 % ($p = 0.01$), but amounts of lactate and alanine remained unchanged. The levels of [3-^{13}C]lactate were lower in both brain regions investigated by 63% ($p = 0.04$) in the HF and 37% ($p = 0.04$) in the cortex. Moreover, [1-^{13}C]glucose content was 38% higher in the HF of mice treated with ALCAR ($p = 0.01$) and a similar trend was seen in cortex (22 % increase, $p = 0.06$). The amounts of [3-^{13}C]alanine were not significantly changed in either brain region.

Following ALCAR administration the amounts of GABA were significantly decreased in the HF by 32 % ($p = 0.01$), but were unchanged in the cortex (Fig. 4), consistent with regional specific metabolic alterations by ALCAR as previously described (Freo et al., 2009; Ori et al., 2002). The amounts of glutamate and glutamine were unaffected in both brain regions. The same was true for the levels of [4-^{13}C]glutamate, [4-^{13}C]glutamine and [2-^{13}C]GABA, the [1-^{13}C]glucose-derived metabolites labeled in the first turn of the TCA cycle via [2-^{13}C]acetyl-CoA. Consequently, the percent ^{13}C enrichment of glutamate and glutamine was unaltered. The same was the case for GABA since the individual values for [2-^{13}C]GABA content in the ALCAR treated animals were smaller than those in the control group, even though this difference was not significant ($p = 0.30$). No alterations in the pyruvate carboxylation to dehydrogenation ratios were observed between control and ALCAR treated animals (results not shown).

3.2. Other metabolites

ALCAR treatment significantly increased the levels of several energy metabolites in the cortex, such as the sum of adenosine nucleotides AMP, ADP, and ATP by 23% ($p = 0.03$), phosphocreatine by 66% ($p = 0.04$), and *myo*-inositol by 30% ($p = 0.02$, Table 1). The ratio of phosphocreatine/creatine (PCr/ratio) was about twofold higher in the cortex of mice treated with ALCAR ($p = 0.03$). The amounts of creatine, succinate, glutathione, choline-containing compounds and N-acetylaspartate (NAA) were unchanged in both brain regions investigated. The levels of nicotinamide adenine dinucleotide (NAD) were not quantifiable in the HF and were unaffected in the cortex.

3.3. Monoamine neurotransmitters

ALCAR administration resulted in a significant 25% increase in the level of NA in the HF ($p = 0.03$), but not in cortex (Fig. 5).

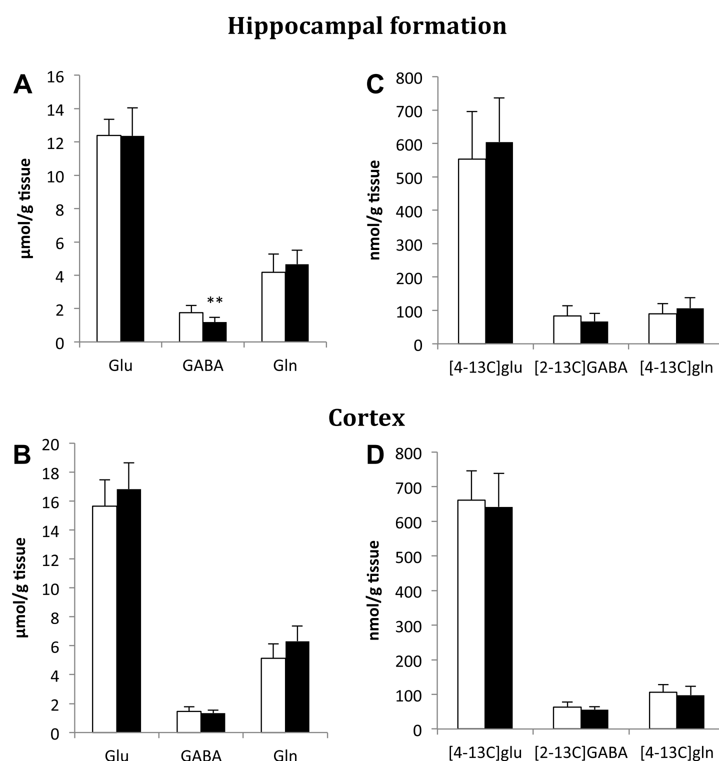

Fig. 4. Amounts of amino acids glutamate, GABA and glutamine (A and B) and ^{13}C-labeled amino acids derived from [1-^{13}C]glucose (C and D) in brain extracts of hippocampal formation and cortex from control (white bars) and ALCAR-treated mice (black bars). Data represent mean ± SD of five control mice and nine ALCAR-treated mice, $^{**}p \leqslant 0.01$, statistically significantly different from control group.

Table 1
Amounts (µmol/g tissue) of metabolites in brain extracts of hippocampal formation and cortex from control and ALCAR-treated mice.

	Hippocampal formation		Cortex	
	Control	ALCAR	Control	ALCAR
AMP + ADP + ATP	1.64 ± 0.97	1.83 ± 1.14	2.48 ± 0.50	3.07 ± 0.35*
Creatine	8.64 ± 0.93	8.02 ± 0.96	9.57 ± 2.06	8.52 ± 1.08
Phosphocreatine	1.62 ± 1.20	2.02 ± 0.72	1.15 ± 0.61	1.91 ± 0.59*
Creatine + phosphocreatine	10.26 ± 1.18	10.05 ± 0.97	10.72 ± 2.45	10.43 ± 0.74
PCr/Cr ratio	0.19 ± 0.15	0.26 ± 0.11	0.12 ± 0.06	0.23 ± 0.09*
NADH	n.d.	n.d.	0.29 ± 0.07	0.36 ± 0.08
Succinate	0.55 ± 0.28	0.63 ± 0.12	0.41 ± 0.10	0.49 ± 0.18
Glutathione	1.30 ± 0.24	1.35 ± 0.30	1.16 ± 0.15	1.32 ± 0.15
Choline[a]	1.72 ± 0.06	1.57 ± 0.18	1.78 ± 0.13	1.82 ± 0.14
Myo-inositol	10.12 ± 2.32	11.04 ± 2.13	5.86 ± 0.80	7.60 ± 1.40*
N-acetylaspartate	5.51 ± 0.34	5.50 ± 0.67	7.36 ± 0.50	7.79 ± 0.97

All metabolite levels were quantified using ^{1}H-NMR spectroscopy, with the exception of glutathione (HPLC) and *myo*-inositol (^{13}C-NMR spectroscopy). Abbreviations: Choline[a]: choline-containing compounds; NADH: nicotinamide adenine dinucleotide; n.d: not determined; PCr/Cr ratio: phosphocreatine/creatine ratio. Data represent mean ± SD of five control mice and nine ALCAR-treated mice, and were analyzed with the Student's *t*-test. $^{*}p \leqslant 0.05$, statistically significant difference from control mice.

Moreover, 5-HT concentrations were significantly increased in the cortex by 20% ($p = 0.005$), with a similar trend in the HF, although without statistical significance (22%, $p = 0.09$). The serotonin turnover rate (5-HIAA/5-HT) was decreased in the cortex from 0.49 to 0.37 ($p = 0.04$), a potential reason for the increase in serotonin levels (Table 2). The following parameters measured were not affected by ALCAR treatment; (1) DA and 5-HIAA amounts in both brain regions and (2) neither DOPAC and HVA levels nor the ratios of DA metabolites to DA, HVA/DA and DOPAC/DA, measures of DA turnover, in cerebral cortex. The amounts of DOPAC and HVA in the HF were below our detection level for quantitation.

4. Discussion

The most important findings of this work are (1) alterations in glucose and lactate metabolism, (2) increases in high energy phosphates and (3) *myo*-inositol, as well as (4) increases in the levels of

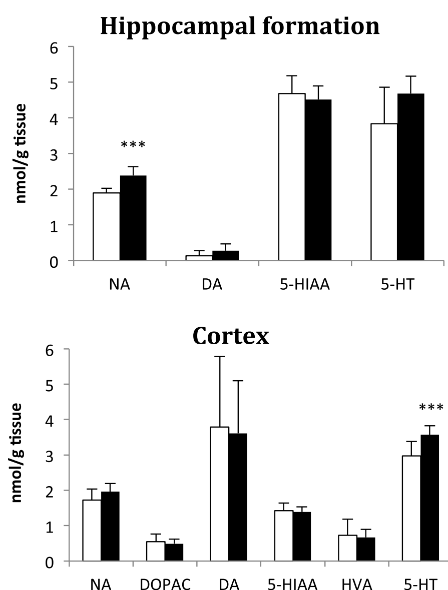

Fig. 5. Amounts (nmol/g tissue) of monoamines and their metabolites in brain extracts of hippocampal formation and cortex from control (white bars) and ALCAR-treated mice (black bars) quantified using HPLC. Abbreviations: Noradrenaline (NA), 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), serotonin (5-HT). Data represent mean ± SD of five control mice and seven ALCAR-treated mice for hippocampal formation, and five control mice and nine ALCAR-treated mice for cortex. $^{***}p \leqslant 0.005$ statistically significantly different from control group, analyzed with the Student's *t*-test.

Table 2
"Turnover" of dopamine and serotonin in brain extracts of hippocampal formation and cortex from control and ALCAR-treated mice.

	Hippocampal formation		Cortex	
	Control (n = 5)	ALCAR (n = 7)	Control (n = 5)	ALCAR (n = 9)
DOPAC/DA	n.d.	n.d.	0.16 ± 0.04	0.15 ± 0.02
HVA/DA	n.d.	n.d.	0.15 ± 0.06	0.20 ± 0.04
5-HIAA/5-HT	1.31 ± 0.43	0.98 ± 0.16	0.49 ± 0.09	0.37 ± 0.05*

The ratios of monoamine neurotransmitters to their metabolites were calculated as a measure for their turnover rates calculated using data from HPLC. Abbreviations: n.d. not determined. Data represent mean ± SD, and were analyzed with the Student's *t*-test. $^{*}p \leqslant 0.05$, statistically significant difference from control mice.

some monoamine neurotransmitters in hippocampus and/or cortex after chronic ALCAR supplementation in mice. The implications of our results are discussed below in relation to previous findings.

4.1. Glucose metabolism

In this study we found that chronic dietary supplementation with ALCAR increased the amount of glucose in both cerebral cortex and HF. Similarly, Ori et al. (2002) and Freo et al. (2009) reported that acute and chronic ALCAR administration increased [^{14}C]2-deoxy-D-glucose labeling in various brain regions. Also, ALCAR treatment did not alter serum concentrations of glucose (Aureli et al., 1998; Freo et al., 2009; Ori et al., 2002) or the uptake of [^{18}F]fluoro-2-deoxy-D-glucose into rat brain slices (Tanaka et al., 2003). This indicates that ALCAR does not alter brain glucose uptake. In agreement with the reported reduced amounts of lactate in the normal adult and aged rat brain after acute ALCAR treatment (Aureli et al., 1990), we also found decreased [3-^{13}C]lactate levels in mice, but no changes in the concentrations of TCA cycle-derived ^{13}C-labeled glutamate, glutamine or GABA. Increased glucose concentration and unchanged TCA cycle activity have also been observed by Nilsen et al. (2011) in mice with reduced α-ketoglutarate dehydrogenase complex activity. Taken together our results indicate that ALCAR administration reduced glucose metabolism to lactate without changing glucose metabolism via the TCA cycle, which lead to higher brain glucose concentrations. Similarly, acute ALCAR treatment counteracted production of lactate in rat and dog models of ischemia (Aureli et al., 1994; Rosenthal et al., 1992).

4.2. Energy metabolites

The levels of the sum of AMP + ADP + ATP, phosphocreatine and the PCr/Cr ratio were significantly increased in the cortex in our mice supplemented with ALCAR compared to control. The fact that the sum of creatine + phosphocreatine was unaltered indicates that the increase in phosphocreatine reflects a larger reservoir of high-energy phosphates. Furthermore, the increased amount of the sum of AMP + ADP + ATP may reflect increased concentration of ATP (see studies below). We could not distinguish the phosphorylation state of adenosine due to resonance overlap in the ^{1}H-NMR spectra. Our findings are in line with several studies demonstrating that ALCAR treatment increased the levels of phosphocreatine and reduced the amount of free organic phosphate in the adult and old rat brain (Aureli et al., 1990), elevated the amounts of phosphocreatine and ATP in a rat model of ischemia (Aureli et al., 1994), prevented ATP depletion in neuroblastoma cells exposed to beta-amyloid (Dhitavat et al., 2002), and ameliorated the decrease of ATP in rat hippocampus after ischemia (Al-Majed et al., 2006). Taken together, the data indicate that ALCAR treatment improves the capacity of the brain to produce high-energy phosphates and reduces anaerobic glucose metabolism. This potential neuroprotective ability may prove to be beneficial in conditions with disturbed energy metabolism.

4.3. Myo-inositol

The level of *myo*-inositol was increased in the cortex of mice supplemented with ALCAR in the present study. Likewise, ALCAR has been shown to prevent *myo*-inositol depletion in a streptomycin induced rat model of diabetic neuropathy (Nakamura et al., 1998; Stevens et al., 1996). *Myo*-inositol is important for the synthesis of PIP2, IP3, DAG and complex signaling phospholipids. If *myo*-inositol is increased due to a decreased synthesis of these molecules, it would have widespread functional consequences. It is also an important osmolyte in the brain and is the most abundant form of inositol (Fisher et al., 2002). *Myo*-inositol has been found to be particularly enriched in astrocytes (Brand et al., 1993). Thus, the increased content of this metabolite appears to reflect an effect of ALCAR on this cell type. The effect on *myo*-inositol suggests that ALCAR can have a positive influence in disorders where water homeostasis is altered.

4.4. Monoamine neurotransmitters

ALCAR supplementation enhanced the amounts of NA and 5-HT in healthy mice. An increased level of NA was detected in the HF, whereas the amount of 5-HT was increased in the cortex, accompanied by a decreased 5-HIAA/5-HT ratio, the latter reflecting reduced serotonin turnover. It is of interest in this context that NA increases oxidative metabolism in cultured astrocytes and freshly dissociated astrocytes express the relevant receptor subtypes (Hertz et al., 2010). In several small trials, ALCAR was found to

be beneficial for patients with depressive disorders and related conditions (Martinotti et al., 2011; Pettegrew et al., 2002; Soczynska et al., 2008; Zanardi and Smeraldi, 2006). The reported increase of 5-HT and NA levels presented here validate further study of these neurotransmitters, their metabolism and receptors in relation to ALCAR and depressive disorders, and may provide rationale for an antidepressant effect of ALCAR.

5. Summary

In conclusion, we report that ALCAR supplementation in healthy mice resulted in improved energy metabolism and sparing of glucose in both HF and cortex. The amounts of the monoamines NA and 5-HT were increased in the HF and cortex respectively. These new insights warrant further studies of ALCAR in clinical settings especially for diseases known to involve energy deficits or monoamine neurotransmitter derangements.

6. Author disclosure

OB. Smeland, TW. Meisingset, K Borges and U. Sonnewald have no conflicts of interest.

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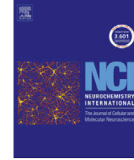

Dietary supplementation with acetyl-L-carnitine in seizure treatment of pentylenetetrazole kindled mice

Olav B. Smeland [1], Tore W. Meisingset [1], Ursula Sonnewald *

Dept. of Neuroscience, Faculty of Medicine, Norwegian University of Science and Technology, Trondheim, Norway

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ABSTRACT

In spite of the availability of new antiepileptic drugs a considerable number of epilepsy patients still have pharmacoresistant seizures, and thus there is a need for novel approaches. Acetyl-L-carnitine (ALCAR), which delivers acetyl units to mitochondria for acetyl-CoA production, has been shown to improve brain energy homeostasis and protects against various neurotoxic insults. To our knowledge, this is the first study of ALCAR's effect on metabolism in pentylenetetrazole (PTZ) kindled mice. ALCAR or the commonly used antiepileptic drug valproate, was added to the drinking water of mice for 25 days, and animals were injected with PTZ or saline three times a week during the last 21 days. In order to investigate ALCAR's effects on glucose metabolism, mice were injected with [1-^{13}C]glucose 15 min prior to microwave fixation. Brain extracts from cortex and the hippocampal formation (HF) were studied using ^{1}H and ^{13}C NMR spectroscopy and HPLC. PTZ kindling caused glucose hypometabolism, evidenced by a reduction in both glycolysis and TCA cycle turnover in both brain regions investigated. Glutamatergic and GABAergic neurons were affected in cortex and HF, but the amount of glutamate was only reduced in HF. Slight astrocytic involvement could be detected in the cortex. Interestingly, the dopamine content was increased in the HF. ALCAR attenuated the PTZ induced reduction in [3-^{13}C]alanine and the increase in dopamine in the HF. However, TCA cycle metabolism was not different from that seen in PTZ kindled animals. In conclusion, even though ALCAR did not delay the kindling process, it did show some promising ameliorative effects, worthy of further investigation.

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1. Introduction

Epilepsy is a diverse group of neurologic seizure disorders; the common denominator being a disposition to excessive and synchronous neuronal activity (Fisher et al., 2005). The life time prevalence of seizure is 2–5%, whereas the prevalence of epilepsy is 5–10 per 1000 in the developed world (Bell and Sander, 2001). After the first unprovoked seizure, 40–50% of patients will experience a recurrence within a two year time period (Berg, 2008) and about one-third of epilepsy patients are pharmacoresistant (Pati and Alexopoulos, 2010) and thus in need of novel therapies.

A reduced ability to utilize glucose is thought to be an important feature of the metabolic disturbances caused by seizure activity (Hagemann et al., 1998; Janigro, 1999). Indeed, ketogenic diet, a treatment for epilepsy, is believed to exert its effect by providing an alternative fuel (Hansen et al., 2009; Melo et al., 2005; Yudkoff et al., 2007). Approximately 50% of children with refractory epilepsy have a significant clinical improvement on this diet (Lefevre and Aronson, 2000). Similarly, alteration of energy metabolism by supplementation with triheptanoin (Willis et al., 2010), had significant anti-convulsive effects in mice.

In light of the reduced ability to metabolize glucose, acetyl-L-carnitine (ALCAR) may serve as an alternative energy source in the brain and thus decrease seizure susceptibility. ALCAR is an endogenous compound involved in the transport of acetyl-moieties across the mitochondrial membrane. ALCAR readily crosses the blood–brain barrier via the organic cation/carnitine transporter OCTN2 (Kido et al., 2001; Parnetti et al., 1992) and carnitine transporters from the OCTN family are present on both neurons and astrocytes (Januszewicz et al., 2010, 2009). Acute ALCAR administration increases phosphocreatine and decreases lactate and inorganic phosphate levels in the rat brain (Aureli et al., 1990). In the mouse brain, chronic supplementation with ALCAR decreases glucose utilization and increases the amounts of high energy phosphates (Smeland et al., in press). Moreover, the ^{13}C

Abbreviations: ALCAR, acetyl-L-carnitine; PTZ, pentylenetetrazole; HF, hippocampal formation; NMR(S), nuclear magnetic resonance (spectroscopy); NAA, *N*-acetyl-aspartate; MTLE, mesial temporal lobe epilepsy.

* Corresponding author. Address: Dept. of Neuroscience, Faculty of Medicine, Olav Kyrresgt. 9, N-7030 Trondheim, Norway. Tel.: +47 73 590492.
E-mail address: ursula.sonnewald@ntnu.no (U. Sonnewald).

[1] These authors contributed equally to this work.

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labeled acetyl moiety of ALCAR may be used in the labeling of amino acids such as glutamate and GABA (Scafidi et al., 2010). Of special interest in regard to a potential anti-convulsant effect, ALCAR protects against excitotoxicity in cell cultures (Forloni et al., 1994). Additionally, ALCAR has been proposed to be preferentially metabolized in GABAergic neurons in the rat brain (Scafidi et al., 2010).

Chemical rodent seizure models have played a vital role in gaining understanding of the basic mechanisms of epileptogenesis, the neurometabolic implications of seizures and antiepileptic treatment. Pentylenetetrazole (PTZ) was first described as a convulsant in 1926 (Hildebrandt, 1926), and its biological and pharmacological profile has been thoroughly investigated (Loscher et al., 1991). As chemoconvulsant, PTZ is used in different acute seizure models (Eloqayli et al., 2003; Green and Murray, 1989; Nutt et al., 1986), in the kindling model (Karler et al., 1989; Kondziella et al., 2002, 2003; Mason and Cooper, 1972) and in models of chronic convulsive seizures (Cremer et al., 2009). Given in appropriate dosage, PTZ is considered to be a model of generalized tonic-clonic seizures (Sarkisian, 2001). Kindling, the phenomenon by which repeated injections of a subconvulsive dose leads to an incremental increase in seizure response as a function of number of injections, is commonly viewed as a model of epileptogenesis and temporal lobe epilepsy (Bertram, 2007; Morimoto et al., 2004). The mechanism of action of PTZ is only partly understood, but an important mechanism is the binding to the picrotoxin-binding site on the GABA-A receptor (Ramanjaneyulu and Ticku, 1984; Squires et al., 1984). Even though the main effect is believed to be a reduction of GABAergic inhibition, PTZ affects several neurotransmitter systems (Maciejak et al., 2010; Szyndler et al., 2010). In previous studies it has been shown that metabolic alterations happen in cortex and hippocampus of animals subjected to one dose of PTZ or to kindling (Carmody and Brennan, 2010; Eloqayli et al., 2003). Eid et al. (2007) have shown that phosphate activated glutaminase expression was increased in hippocampus of patients with chronic MTLE and Petroff et al. (2002) showed reduced glutamate glutamine cycling in the same area. Furthermore, cortex and the HF are the brain regions which are most prone to primary neurohistopathologic change due to status epilepticus in humans and animal models (Drislane, 2005). Therefore we chose to study metabolic alterations in these areas in animals that had experienced repeated generalized tonic-clonic convulsions. It should be stressed that even though PTZ injection has been shown to cause changes in neuronal architecture in the hippocampus (Cavazos et al., 2004) it does not induce neuronal death (Planas et al., 1994; Valente et al., 2004).

In this context we asked if ALCAR supplementation could affect seizure activity and induce metabolic alterations in PTZ kindled mice. In order to address the question we used [1-^{13}C]glucose in combination with ^{1}H and ^{13}C ex vivo nuclear magnetic resonance spectroscopy (NMRS) and HPLC and determined regional brain energy metabolism, as well as the concentrations of dopamine, serotonin and noradrenaline in the early postictal period in PTZ kindled mice.

2. Materials and methods

2.1. Chemicals

[1-^{13}C]glucose (99% ^{13}C) and D_2O (99.9%) were purchased from Cambridge Isotope Laboratories (Woburn, MA, USA), ethylene glycol from Merck (Darmstad, Germany), and acetyl-L-carnitine (ALCAR), valproate and pentylenetetrazole (PTZ) from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of the purest grade available from local commercial sources.

2.2. Animal model

The Norwegian National Animal Research Authority and the local ethics committee approved the experimental procedures. Male NMRI mice ($n = 28$; Taconic, Ejby, Denmark) were used in the experiment. The mice had an average weight of 25 ± 0.7 g at the start of the study. The animals were randomly separated into four groups: (1) chronically saline injected mice (control mice), (2) PTZ-kindled mice, (3) ALCAR-supplemented PTZ-kindled mice (PTZ–ALCAR mice) and (4) valproate-treated PTZ-kindled mice (PTZ–valproate mice). In the PTZ-kindled group one mouse died from status epilepticus, and one was found moribund. One mouse died in the PTZ–ALCAR group during status epilepticus. All animals were maintained under standard laboratory conditions on a 12/12-h light/dark cycle (lights on at 7AM), at a constant temperature of 22 °C and a humidity of 60%. Animals were housed in individual cages with free access to food and water. The mice were acclimatized to the above conditions for 1 week before the start of the experiments.

The ALCAR groups received a 1.5 g/L solution of ALCAR as drinking water (pH adjusted to 6), and were allowed to drink *ad libitum*. PTZ-ALCAR mice drank approximately 10 ml per day (data not shown), which would provide a daily ALCAR dose of approximately 500 mg/kg body weight. The PTZ–valproate group received a 3.3 g/L solution of valproate in water (pH 7.5), and was allowed to drink *ad libitum*. Valproate receiving mice typically drank approximately 8 ml per day (data not shown), which would provide a daily valproate dose of approximately 900 mg/kg body weight. The control mice and PTZ-control mice typically drank 7.5 ml water per day. ALCAR and valproate were supplemented in the drinking water for 4 days before the experimental procedure with PTZ started. At the end of the study, the mean weights of the groups were: control mice: 36.5 ± 0.7 g; PTZ-control mice: 34.2 ± 1.6 g; PTZ–ALCAR mice: 35.7 ± 1.1 g; and PTZ–valproate mice: 32.1 ± 0.7 g. The PTZ–valproate mice had a significantly lower weight compared to control mice and PTZ–ALCAR mice.

Mice were intraperitoneally injected with PTZ (43 mg/kg, PTZ-kindled, PTZ–ALCAR and PTZ–valproate groups) or saline (0.1 mg/kg, control group) three times a week for three weeks while they received normal drinking water or water supplemented with ALCAR or valproate. After PTZ injection mice were observed for 30 min for latency to clonic convulsions and the severity of convulsions was scored according to a modified Racine scale (0 = no response, 1 = 1–3 myoclonic jerks and/or facial twitching and/or axial waves going though the body, 2 = more than three myoclonic jerks, 3 = clonic convulsion with forelimb clonus without loss of postural control, 4 = clonic convulsion with loss of postural control, turning to the side and/or rearing, 5 = clonic convulsion with loss of righting reflex and/or bouncing, two or more clonic convulsions, tonic convulsion or status epilepticus). The mice were randomized before PTZ injections, and the observers were blinded to the identity of the animal.

The mice received a final dose of PTZ or saline and were, 15 min later, intraperitoneally injected with [1-^{13}C]glucose (543 mg/kg, 0.3 M solution). This protocol of glucose injection leads to blood glucose levels well within the normoglycemic range (8.2 ± 0.5 mmol/L; Hassel et al., 1995). 15 min after the glucose injection, the mice were subjected to microwave fixation at 4 kW for 1.7 s (Model GA5013, Gerling Applied Engineering, Modesto, CA, USA). The time interval of 15 min after glucose injection was chosen based on our previous experiments (Hassel et al., 1995). The brains were removed and the cortex and hippocampal formation (HF) were dissected and stored at −80 °C till extraction. The HF included the dentate gyrus, hippocampus proper, subiculum, but not entorhinal cortex. The tissue samples were homogenized in 0.7% perchloric acid using a Vibra Cell sonicator (Model VCX

750, Sonics & Materials, Newtown, CT, USA). Thereafter the samples were centrifuged at 4400 g and 4 °C for 5 min. The procedure was repeated, the supernatants were pooled and samples for monoamine HPLC analysis were removed. Thereafter, pH was adjusted to 6.5–7.5 with 1 M KOH followed by centrifugation and lyophilization.

2.3. High performance liquid chromatography (HPLC)

To determine the total amount of glutathione (reduced plus oxidized) the samples were analyzed using a Hewlett Packard 1100 System (Agilent Technologies, Palo Alto, CA, USA) with fluorescence detection, after derivatization with *o*-phtaldialdehyde (Geddes and Wood, 1984). The components were separated with a ZORBAX SB-C18 (4.6 × 150 mm, 3.55 μm) column from Agilent using 50 mM sodium phosphate buffer (pH 5.9) with 2.5% tetrahydrofurane and methanol (98.75%) with tetrahydrofurane (1.25%) as eluents. The amount of glutathione was quantified by comparison with a standard curve derived from a standard solution of glutathione run repeatedly with 15 samples intervals.

To determine the amounts of monoamines**,** the samples were analyzed using an Agilent 1200 system (Agilent, USA) with an electrochemical detector (Coulochem III, ESA, Sunnyvale, CA, USA). The components were separated with an Eclips XDB-C18 column (4.6 × 150 mm, 5 μm, Agilent) with an aqueous mobile phase (0.90 mL/min) containing 90 mM NaH2PO4, 50 mM citric acid, 0.1 mM EDTA, 0.5 mM octanesulfonic acid and 7% methanol solution. The amounts of monoamines were quantified by comparison with a standard curve derived from standard solutions of monoamines run after every 15th sample.

2.4. ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy

Lyophilized samples were dissolved in 200 μL of D_2O containing 0.01% ethylene glycol as an internal standard for quantification. pH was re-adjusted to 6.5–7.5, and samples were transferred into 5 mm Shigemi NMR microtubes (Shigemi Inc., Allison Park, PA, USA).

The HF samples were analyzed using a BRUKER DRX-600 spectrometer (BRUKER Analytik GmbH, Rheinstetten, Germany), while the cortex samples were analyzed using a BRUKER DRX-500 spectrometer. The spectra were recorded at 25 °C. ^{1}H NMR spectra were acquired on the same instruments with the following parameters: pulse angle of 90°, acquisition time 1.36 s and relaxation delay 10 s. The number of scans was 128 and 1024 for cortical and hippocampal extracts, respectively. Water suppression was achieved by applying a low-power pre-saturation pulse at the water frequency. Proton decoupled ^{13}C NMR spectra were obtained using a 30° pulse angle and 30 kHz spectral width with 64 K data points employing an acquisition time of 1.08 s and a relaxation delay of 0.5 s. The number of scans needed to obtain an appropriate signal to noise ratio was typically 25,000 for the cortex samples and 35,000 for the HF samples.

Relevant peaks in the spectra were identified and integrated using XWINNMR software (BRUKER BioSpin GmbH, Rheinstetten, Germany). The total amounts and ^{13}C labeling of metabolites were

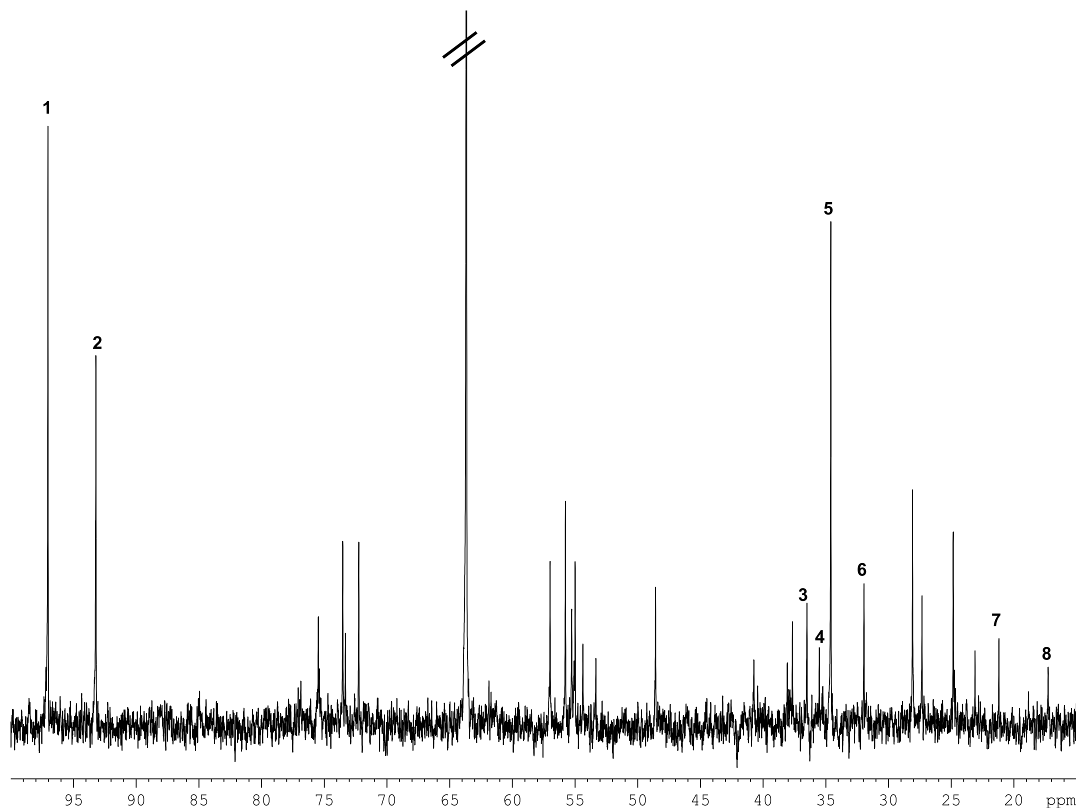

Fig. 1. Typical ^{13}C NMR spectrum from cerebral cortex extract of a PTZ–ALCAR mouse. Peak assignment: 1:β-glucose C-1; 2: α-glucose C-1; 3: aspartate C-3; 4: GABA C-2; 5: glutamate C-4; 6: glutamine C-4; 7: lactate C-3; 8: alanine C-3.

quantified from the integrals of the peak areas using ethylene glycol as an internal standard. Correction for natural abundance, nuclear Overhauser enhancement and relaxation effects relative to the internal standard were applied to all relevant resonances.

2.5. Labeling patterns of compounds from metabolism of [1-^{13}C]glucose

Fig. 1 shows a typical ^{13}C NMR spectrum. To interpret the ^{13}C incorporation results it is necessary to analyze the metabolism of [1-^{13}C]glucose (Fig. 2). via glycolysis, [1-^{13}C]glucose is converted to [3-^{13}C]pyruvate, which can be further converted to [3-^{13}C]alanine, [3-^{13}C]lactate or [2-^{13}C]acetyl-CoA. [2-^{13}C]acetyl-CoA may enter the tricarboxylic acid (TCA) cycle through condensation with oxaloacetate to form [4-^{13}C]citrate. Eventually the TCA cycle intermediate [4-^{13}C]α-ketoglutarate is formed, which is the precursor of [4-^{13}C]glutamate. Thereafter, [4-^{13}C]glutamate may be transformed to [4-^{13}C]glutamine in astrocytes due to the astrocyte specific localization of glutamine synthetase (Norenberg and Martinez-Hernandez, 1979) and to [2-^{13}C]GABA in GABAergic neurons. If [4-^{13}C]α-ketoglutarate is further converted in the TCA cycle, labeled oxaloacetate will be formed which can be transaminated to [2-^{13}C]aspartate or [3-^{13}C]aspartate. Further cycling of labeled metabolites give rise to different labeling patterns in amino acids (Alvestad et al., 2008).

2.6. Data analysis

Statistical analysis of metabolites and seizure latency was performed using one-way ANOVA followed by a least significant difference post hoc test, were $p < 0.05$ was regarded as significant. Analysis of seizure severity was performed using a Kruskal–Wallis test (nonparametric ANOVA) followed by a Mann–Whitney *U* test. All data are represented as mean ± S. Due to technical problems, the number of analyses varied between analytical methods.

3. Results

The objective of the present study was to investigate possible beneficial effects of ALCAR in a PTZ kindling model. The progression of kindling is presented in Fig. 3A. Valproate delayed the progression of kindling in the initial stage of the experiment evidenced by a significantly lower Racine score on injection day 3. However, valproate did not delay the occurrence of full kindling. PTZ–ALCAR mice had the same kindling progression as PTZ kindled mice. Latency to clonic convulsions was not affected by valproate or ALCAR (Fig. 3B).

Microwave fixation was performed on injection day 10, thus all metabolic analyses were at a time point when seizures were at a maximum and there were no differences in Racine score between groups. This provided an opportunity to assess ALCAR's metabolic effects on animals subjected to repeated generalized tonic-clonic convulsions. In the HF, four groups were compared: (1) control mice, (2) PTZ-kindled mice, (3) PTZ–ALCAR mice and (4) PTZ–valproate mice. In cortex, three groups were compared: (1) control mice, (2) PTZ-kindled mice and (3) PTZ–ALCAR mice.

Analyses of ^{1}H NMR spectra from cerebral cortex and HF enabled us to quantify many metabolites. Total amount of glucose, lactate and alanine are presented in Table 1. Together with acetyl-CoA, both lactate and alanine are derived from the end-product of glycolysis, pyruvate. In cortex, total amount of glucose was increased in all PTZ-kindled animals compared to control, whereas

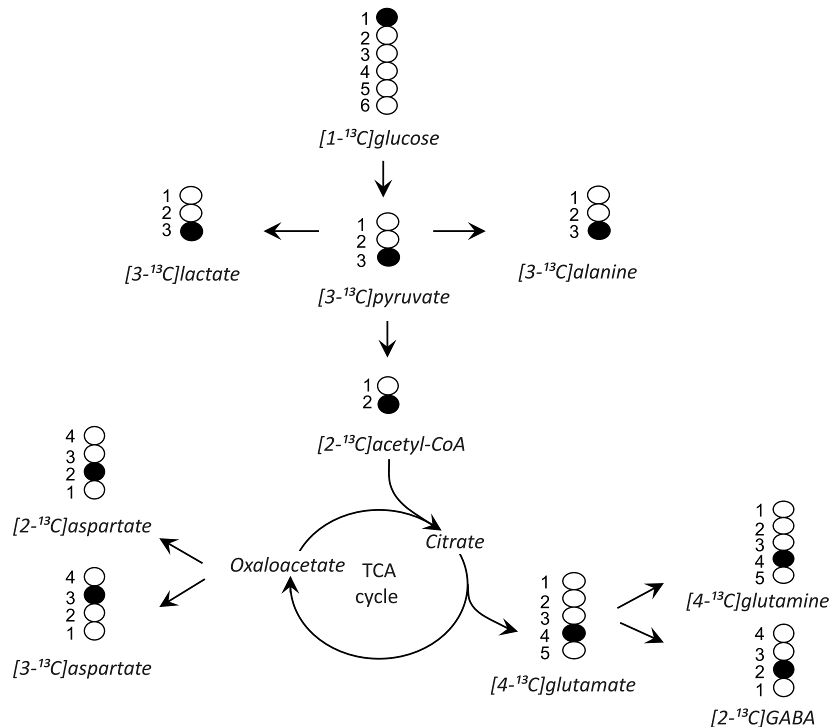

Fig. 2. Schematic representation of isotopomers derived from [1-^{13}C]glucose. Black circles indicate ^{13}C labeling. Only the first turn of the TCA cycle is illustrated.

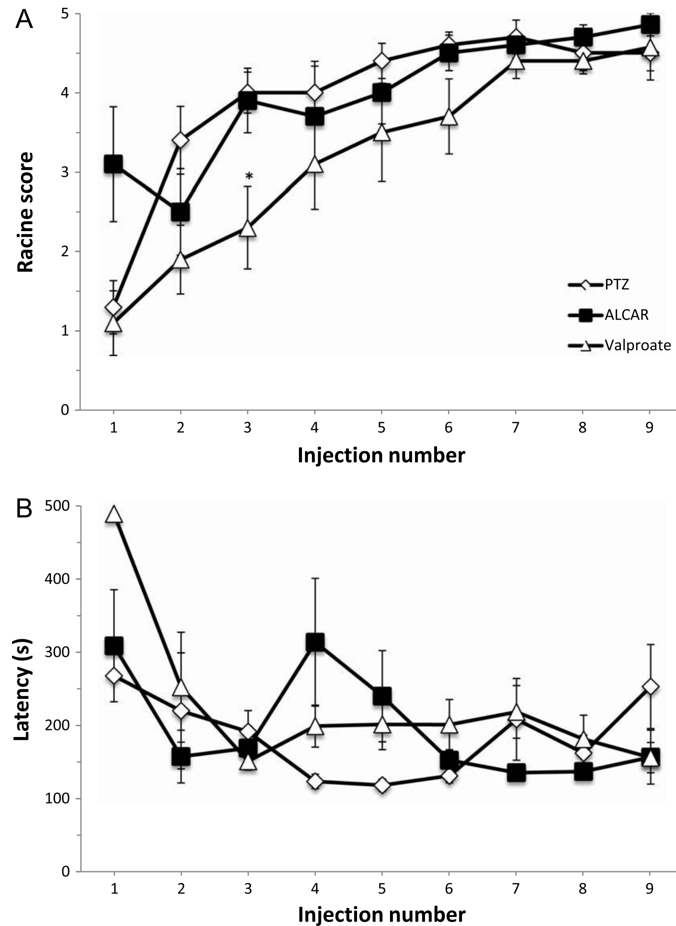

Fig. 3. Development of kindling in pentylenetetrazole (PTZ)-kindled mice, PTZ-kindled mice supplemented with acetyl-L-carnitine (ALCAR), and PTZ-kindled mice treated with valproate. Mice were i.p. injected with 43 mg/kg PTZ three times a week for three weeks. (A) Severity of seizures as a function of injections. (B) Latency (in seconds) to first clonic convulsion. Analysis of seizure severity was performed using a Kruskal–Wallis test followed by a Mann–Whitney *U* test. Latency was analyzed using a one-way ANOVA followed by a LSD post hoc test. Data are represented as mean ± SEM, and the superscripts (a, b) indicate a statistically significant difference to the PTZ-group and the PTZ–ALCAR group respectively ($p < 0.05$).

Table 1
Amounts, ^{13}C-labeling and percent ^{13}C enrichment of glucose, lactate and alanine in brain extracts of hippocampal formation and cortex from control mice, PTZ-kindled mice, PTZ–ALCAR treated mice, and PTZ–valproate treated mice.

nmol/g tissue	Hippocampal formation				Cortex		
	Control[A] $n = 5$	PTZ $n = 5$	PTZ-ALCAR $n = 5$	PTZ-valproate $n = 6$	Control $n = 5$	PTZ $n = 5$	PTZ–ALCAR $n = 4$
Glucose	2410.1 ± 315.1	3053.3 ± 174.1	3248.3 ± 354.0	3785.2 ± 298.8[a]	3042.1 ± 247.6	5205.8 ± 317.6[a]	4948.2 ± 235.0[a]
[1-^{13}C]glucose	601.0 ± 79.0	702.0 ± 49.7	649.0 ± 31.4	789.2 ± 42.1	553.4 ± 33.4	687.0 ± 94.3	606.0 ± 23.2
^{13}C enrichment glucose	25.9 ± 3.7	23.2 ± 1.8	20.9 ± 2.4	21.1 ± 1.4	18.6 ± 1.5	13.2 ± 1.7[a]	12.3 ± 0.8[a]
Lactate	4999.9 ± 1280.1	2167.2 ± 163.0[a]	3309.3 ± 530.6	2804.1 ± 137.3[a]	4548.2 ± 570.4	2992.1 ± 329.4	3596.5 ± 552.6
[3-^{13}C]lactate	320.2 ± 120.0	60.2 ± 9.2[a]	121.0 ± 15.1[a]	86.1 ± 18.4[a]	170.1 ± 34.4	85.5 ± 12.5[a]	82.7 ± 21.2[a]
^{13}C enrichment lactate	5.9 ± .10	2.8 ± 0.5	4.3 ± 1.0	3.0 ± 0.6	3.7 ± 0.5	3.0 ± 0.6	2.2 ± 0.3
Alanine	1004.0 ± 50.5	742.4 ± 54.2	909.1 ± 140.2	809.9 ± 134.6	1042.7 ± 84.8	1130.4 ± 204.2	1192.5 ± 119.3
[3-^{13}C]alanine	68.7 ± 13.3	24.2 ± 1.5[a]	57.9 ± 8.3[b]	37.9 ± 12.7	53.6 ± 9.4	36.2 ± 1.3	33.2 ± 6.0
^{13}C enrichment alanine	6.8 ± 1.3	3.4 ± 0.4	6.5 ± 0.8	4.0 ± 1.3	5.0 ± 0.5	3.7 ± 0.8	2.9 ± 0.3

Amounts of metabolites were calculated using data from ^{1}H- and ^{13}C-NMRS. See Materials and methods for details. Data represent mean ± SEM, and the superscripts (a,b) indicate a statistically significant difference to the control group, the PTZ group and the PTZ–ALCAR group respectively ($p < 0.05$).

[A] For the glucose data the control group included only four mice. Control data from Smeland et al. (epub).

only PTZ–valproate mice had a significant increase in the HF. Lactate levels in the HF of PTZ-kindled mice and PTZ–valproate mice were significantly decreased, which can indicate reduced glycolytic activity. Amounts of alanine were unaltered in both brain regions.

[1-^{13}C]glucose was administered 15 min. before microwave fixation and the labeling from glucose and glycolysis products was determined by ^{13}C NMRS. Table 1 presents levels of [1-^{13}C]glucose, [3-^{13}C]lactate and [3-^{13}C]alanine. The amount of [1-^{13}C]glucose was unaltered in both brain areas in all groups. [3-^{13}C]lactate content was decreased in both brain regions in all PTZ-kindled animals. [3-^{13}C]alanine content was unaltered in cortex, but was significantly decreased in PTZ kindled mice in the HF. Interestingly, the amount was significantly higher in PTZ–ALCAR mice compared to PTZ kindled mice.

PTZ–valproate mice had a significantly lower weight at the end of the experiment. This weight difference did not affect glucose availability, since glucose was administered according to weight. Furthermore, glucose enrichment was similar in HF in all mice. In Table 2 we list metabolites related to energy status: AMP + ADP + ATP (adenosine monophosphate + adenosine diphosphate + adenosine triphosphate), creatine, phosphocreatine and NADH; the TCA cycle intermediate succinate; the osmolytes taurine and *myo*-inositol; choline containing compounds; *N*-acetyl aspartate (NAA) and the antioxidant glutathione (the sum of the reduced and oxidized forms). In the cortex of PTZ-kindled mice total amount of succinate was significantly decreased, whereas that of *myo*-inositol was significantly elevated. In the HF, valproate treatment increased the glutathione level, whereas the levels of choline containing compounds were significantly decreased in PTZ-kindled mice and in PTZ–ALCAR mice. The amount of the monoamines dopamine, noradrenaline and serotonin are also presented in Table 2. Dopamine concentration was increased in HF of PTZ-kindled and PTZ–valproate mice compared to control.

The amino acids essential for glutamatergic and GABAergic neurotransmission: glutamate, glutamine, GABA and aspartate are presented in Fig. 4 (cortex) and Fig. 5 (HF). In cortex, total amounts of glutamate, glutamine, GABA and aspartate were not altered. However, labeling from [1-^{13}C]glucose in [4-^{13}C]glutamate, [4-^{13}C]glutamine, [2-^{13}C]GABA and [3-^{13}C]aspartate was significantly decreased in PTZ and PTZ–ALCAR groups compared to control. Percent enrichment in glutamate, glutamine and aspartate was similarly decreased. In the PTZ kindled mice, percent enrichment in GABA was decreased compared to control, whereas in PTZ–ALCAR mice, percent enrichment in GABA was decreased compared to control and PTZ kindled mice. GABA content was similar between PTZ and PTZ–ALCAR mice, whereas in [2-^{13}C]GABA content there was a reduction of 32% ($p = 0.08$) between the two groups. In HF, total amount of glutamate was decreased in all PTZ-kindled animals. Percent ^{13}C enrichment was unaltered, whereas [4-^{13}C]glutamate and [2-^{13}C]GABA content was decreased in all PTZ-kindled animals.

4. Discussion

We investigated the antiseizure capability and metabolic effects of ALCAR on mice subjected to PTZ kindling using a similar experimental protocol of PTZ injection as Hansen et al. (2009). This group demonstrated that ketogenic diet was able to delay kindling development. We compared the effects of the dietary supplement ALCAR to those of the well-established antiepileptic drug valproate, which has been shown to delay kindling (Abdallah, 2010). In the present study, valproate did delay the progression of kindling slightly in the initial phase, but, in agreement with Ohno et al. (2010) did not delay the occurrence of full kindling. To our disappointment the PTZ–ALCAR mice showed the same kindling progression and latency to clonic convulsions as those that received PTZ alone.

PTZ-kindling affected glucose metabolism in the present study in agreement with earlier reports on postictal hypometabolism (Cooper et al., 2001; Hagemann et al., 1998). Valproate reduces TCA turnover in mice (Johannessen et al., 2001) and, in concurrence, global CMRglc in epilepsy patients and healthy volunteers receiving valproate is decreased (Gaillard et al., 1996; Leiderman et al., 1991). In addition ALCAR has been shown to decrease glucose metabolism (Smeland et al., in press).

4.1. The effect of PTZ kindling on glucose metabolism

Increased glucose content was detected in cortex of all PTZ kindled mice, indicating reduced glucose utilization (Lei et al., 2010). In the HF, decreased glucose utilization was only statistically significant in PTZ–valproate mice. However, the reduction in lactate concentration and amount of [3-^{13}C]lactate in PTZ-kindled mice together with the reduction of [3-^{13}C]alanine content, did point to hippocampal glucose hypometabolism in the PTZ-kindled mice.

Table 2
Amounts (μmol/g tissue) of metabolites in brain extracts of hippocampal formation and cortex from control, PTZ-kindled, PTZ–valproate and PTZ–ALCAR mice.

	Hippocampal formation				Cortex		
	Control $n = 5$	PTZ $n = 5$	PTZ-ALCAR $n = 6$	PTZ-valproate $n = 5$	Control $n = 5$	PTZ $n = 5$	PTZ-ALCAR $n = 5$
AMP + ADP + ATP	1.6 ± 0.4	1.8 ± 0.2	1.4 ± 0.3	1.7 ± 0.3	2.5 ± 0.2	2.6 ± 0.3	2.4 ± 0.3
Creatine	8.6 ± 0.4	7.5 ± 0.7	8.4 ± 0.4	7.8 ± 0.6	9.6 ± 0.9	9.2 ± 0.6	9.4 ± 0.4
Phosphocreatine	1.6 ± 0.5	2.2 ± 0.4	1.1 ± 0.2	1.9 ± 0.4	1.1 ± 0.3	1.4 ± 0.2	1.1 ± 0.2
NADH	n.q	n.q	n.q	n.q	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
NAA	4.6 ± 0.5	3.7 ± 0.3	3.7 ± 0.2	4.1 ± 0.4	8.9 ± 0.7	8.9 ± 0.5	9.2 ± 0.6
Succinate	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.9 ± 0.3	0.4 ± 0.2	0.2 ± 0.0$^{\mathbf{a}}$	0.3 ± 0.1
Taurine	11.1 ± 0.6	10.3 ± 0.5	10.5 ± 0.6	10.7 ± 0.4	13.4 ± 1.0	12.0 ± 1.0	13.9 ± 0.9
Glutathione	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.9 ± 0.1$^{\mathbf{abc}}$	1.2 ± 0.1	1.3 ± 0.0	1.3 ± 0.1
Myo-inositol^{A}	10.1 ± 1.0	11.3 ± 0.6	9.2 ± 1.3	10.8 ± 1.0	5.9 ± 0.4	8.3 ± 0.9$^{\mathbf{a}}$	7.7 ± 0.1
Choline containing compounds	1.7 ± 0.0	1.3 ± 0.2$^{\mathbf{a}}$	1.3 ± 0.0$^{\mathbf{a}}$	1.5 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
Dopamine	0.2 ± 0.1	0.7 ± 0.1$^{\mathbf{a}}$	0.5 ± 0.1	0.8 ± 0.1$^{\mathbf{ac}}$	3.8 ± 0.9	5.4 ± 0.7	3.3 ± 0.5
Noradrenaline	1.9 ± 0.1	1.6 ± 0.1	1.6 ± 0.2	1.6 ± 0.2	1.7 ± 0.1	1.6 ± 0.1	1.5 ± 0.1
Serotonin	3.8 ± 0.5	4.5 ± 0.2	3.8 ± 0.3	4.5 ± 0.4	3.0 ± 0.2	3.7 ± 0.2	3.4 ± 0.4

Mice received ALCAR or valproate in the drinking water for 25 days, and two groups were injected with PTZ intraperitoneally three times a week (for details see Materials and methods). All metabolites were obtained using ^{1}H NMRS, with the exception for glutathione, which was measured by HPLC, and *Myo*-inositol, which was measured by ^{13}C NMRS.

Abbreviations: n.q: not quantifiable; NAA: *N*-acetyl aspartate. Data represent mean ± SEM, and the superscripts (a, b, c) indicate a statistically significant difference to the control group, the ALCAR group and the PTZ group, respectively ($p < 0.05$). Control data from Smeland et al. (epub).

^{A} The PTZ–ALCAR group included only five mice for the hippocampal formation, and four mice for the cortex.

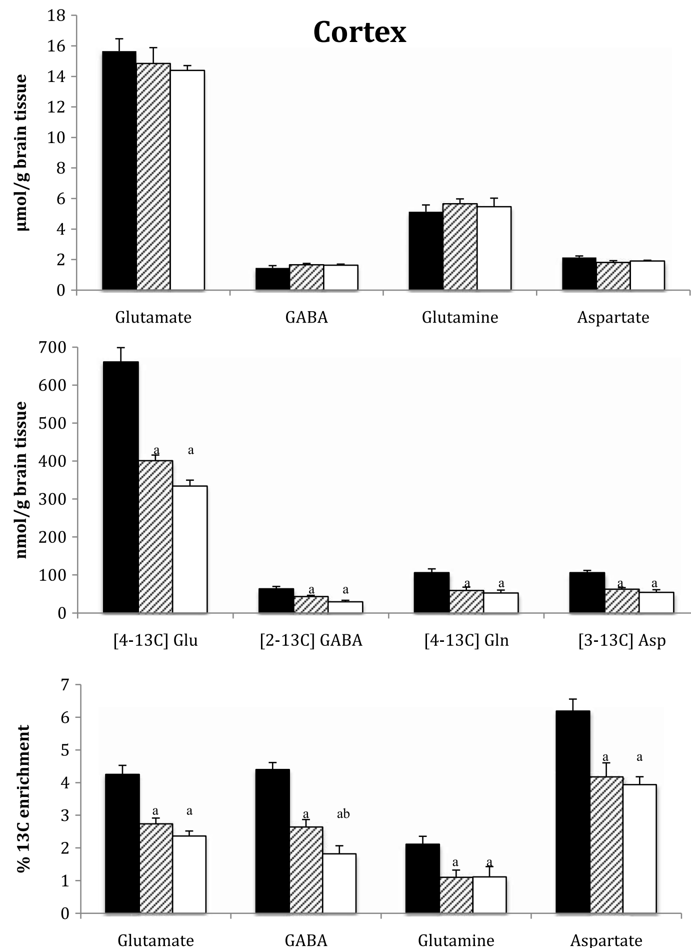

Fig. 4. Amounts and labeling of glutamate, glutamine, GABA and aspartate in the cortex of control mice ($n = 5$, black bars), PTZ-kindled mice ($n = 5$, striped bars), and PTZ–ALCAR treated mice ($n = 4$, white bars). (A) The amounts of metabolites (µmol/g tissue) were obtained by ^{1}H NMRS. (B) The amounts of ^{13}C-labeled metabolites (nmol/g tissue) were obtained by ^{13}C NMRS. (C) The percent ^{13}C-enrichments of amino acids were calculated using data from ^{1}H- and ^{13}C-NMRS. The results are expressed as mean ± SEM, and the superscripts (a, b) indicate a statistically significant difference to the control group and the PTZ-group respectively ($p < 0.05$). Control data from Smeland et al. (in press).

Using an experimental protocol similar to ours, Yudkoff et al. (2003) concluded that glucose metabolism was enhanced as a consequence of a seizure-induced energy deficit. The discrepancy between the hypometabolism detected by us and the hypermetabolism reported by Yudkoff et al. (2003) may be due to differences in interpretation and the fact that we analyzed the entire cortex cerebri whereas Yudkoff et al. (2003) analyzed the forebrain. Alterations in the rate of glucose metabolism have also been assessed in cell culture studies of astrocytes, where glycolysis was decreased by short-term incubation with PTZ, whereas long-term incubation caused increased glycolysis (Qu et al., 2005). The present data do not allow a conclusive statement regarding in which compartment(s) glycolysis is altered.

Not only glycolysis but also TCA cycle activity was affected in the PTZ-kindled mice. Pyruvate, the end point of glycolysis, is the precursor for lactate, alanine and acetyl-CoA. In cortex in the present study, ^{13}C enrichment in glucose was decreased but that of lactate and alanine was unchanged and thus it is likely that also ^{13}C enrichment in acetyl-CoA was unchanged. Taken together with a decrease in succinate level, the reduction in ^{13}C enrichment and amount of [4-^{13}C]glutamate, [2-^{13}C]GABA, [4-^{13}C]glutamine and [3-^{13}C]aspartate suggests a reduced TCA cycle turnover in the early post-ictal phase in glutamatergic and GABAergic neurons. In HF ^{13}C enrichment in glucose was not altered. The amounts of [4-^{13}C]glutamate and [2-^{13}C]GABA were decreased together with a decrease in the amount of glutamate indicating involvement of both glutamatergic and GABAergic neurons. The observed decreased TCA cycle turnover is in agreement with results from a study using cultured cerebellar granule neurons, where reduced TCA cycle turnover was detected (Eloqayli et al., 2002). Also after a single injection of PTZ, TCA-cycle activity was reduced, though mostly in glutamatergic neurons, not in astrocytes (Eloqayli et al., 2003). Glial involvement has been shown in PTZ-kindling experiments using senescent-accelerated mice (Kondziella et al., 2003). The increase in *myo*-inositol in cortex in the present study points towards glial involvement since most *myo*-inositol is localized in

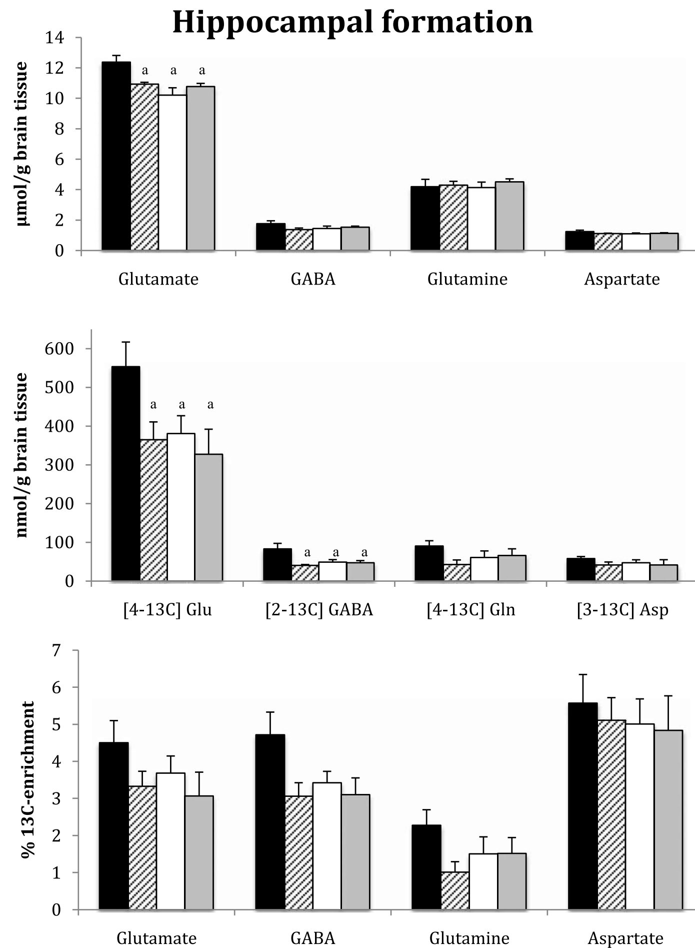

Fig. 5. Amounts and labeling of glutamate, glutamine, GABA and aspartate in the hippocampal formation of control mice ($n = 5$, black bars), PTZ-kindled mice ($n = 5$, striped bars), PTZ–ALCAR treated mice ($n = 5$, white bars), and PTZ–valproate treated mice ($n = 6$, gray bars). A: The amounts of metabolites (µmol/g tissue) were obtained by ^{1}H NMRS. (B) The amounts of ^{13}C-labeled metabolites (nmol/g tissue) were obtained by ^{13}C NMRS. (C) The percent ^{13}C-enrichments of amino acids were calculated using data from ^{1}H- and ^{13}C-NMRS. The results are expressed as mean ± SEM, and the superscripts (a, b, c) indicate a statistically significant difference to the control group, the PTZ group and the PTZ–ALCAR group respectively ($p < 0.05$). Control data from Smeland et al. (in press).

astrocytes (Brand et al., 1993; Meisingset et al., 2010). However, even though the reduction in [4-^{13}C]glutamine labeling may suggest astrocytic involvement in cortex, this reduction could also be due to a disturbance in neuronal release of [4-^{13}C]glutamate. The latter explanation is supported by the unaltered total amount of glutamine in both HF and cortex. Indeed, in HF, unaltered [4-^{13}C]glutamine labeling indicates unaffected mitochondrial metabolism in astrocytes.

Amounts of the neurotransmitters glutamate, GABA and aspartate were not altered in the present study, except for a reduction in glutamate in HF. Similarly, Maciejak et al. (2010) demonstrated a down regulation of glutamate levels in the hippocampus after administration of a single dose of PTZ, but this effect was not seen after kindling. These authors have shown that homeostasis of excitatory and inhibitory amino acids during the PTZ kindling process is complex, with great variations between brain regions. Decrease of glutamate concentration is also seen in the kainate and lithium-pilocarpine models of temporal lobe epilepsy (Alvestad et al., 2008; Melo et al., 2005), indicating that the observed down regulation of glutamate content is not PTZ specific, but possibly due to seizures.

4.2. The effects of ALCAR on glucose metabolism in PTZ kindling

It is well established that the uptake of ALCAR into brain tissue is efficient (Kuratsune et al., 1997; Scafidi et al., 2010). Since OCTN transporters are found on both astrocytes and neurons, ALCAR may be internalized in both compartments. Moreover, chronic ALCAR supplementation does not alter the blood glucose level (Freo et al., 2009; Ori et al., 2002). Thus, the PTZ–ALCAR mice had the same glucose availability as the other PTZ kindled mice.

In the HF, PTZ kindling reduced the amounts of lactate and [3-^{13}C]alanine compared to control mice. Interestingly, ALCAR supplementation normalized these PTZ induced alterations, indicating a positive effect of ALCAR on glucose metabolism in this region. Importantly, since PTZ-kindled mice and PTZ–ALCAR mice had a

similar kindling development, such differences are a result of ALCAR specific effects on metabolism and not a consequence of differences in seizure activity.

Catabolism of ALCAR can lead to mitochondrial acetyl-CoA formation which could dilute the amount of [1-^{13}C]glucose derived [2-^{13}C]acetyl-CoA in our study. The fact that the amount of [3-^{13}C]alanine was normalized in PTZ–ALCAR mice compared to PTZ-kindled mice can be interpreted as a partial redirection of pyruvate because the TCA cycle is also supplied with the acetyl moiety of ALCAR. However, we have previously shown that only the conversion of glucose into lactate, i.e. anaerobic glycolysis, is affected by the presence of ALCAR while the conversion of glucose into pyruvate which via acetyl CoA subsequently enters the TCA cycle is not affected by ALCAR (Smeland et al., in press). Indeed, also in the present study, the PTZ–ALCAR mice showed the same reduction in ^{13}C label in glutamate, glutamine, GABA and aspartate as PTZ-kindled mice. However, ^{13}C enrichment of GABA in cortex of PTZ–ALCAR mice was lower compared to that of PTZ-kindled mice. No alterations were detected in GABA levels in PTZ–ALCAR mice, even though ALCAR has been shown to induce a decrease in the GABA concentration in HF of healthy mice (Smeland et al., in press). In light of the unaltered GABA content the reduction in ^{13}C enrichment may suggest incorporation of the unlabeled acetyl-moiety of ALCAR into GABA, which then would dilute ^{13}C-label from [1-^{13}C]glucose incorporation. This is in compliance with the results of Scafidi et al. (2010) which demonstrated increased ^{13}C enrichment of GABA after [2-^{13}C]ALCAR supplementation. As the authors discussed, this high ^{13}C enrichment of GABA could indicate a preferential metabolism of ALCAR in GABAergic neurons. Furthermore, ALCAR did not alter the PTZ-induced reduction in glutamate content in the HF. ALCAR failed to reverse the major PTZ effects on TCA cycle metabolites.

In a previous study, ALCAR has shown the capability to increase amounts of phosphocreatine and adenosine phosphates (Smeland et al., in press). In the present study, however, the concentrations of high-energy phosphates were similar in PTZ–ALCAR mice compared to PTZ-kindled mice and control mice. In cortex ALCAR also had a normalizing effect on the level of *myo*-inositol, suggesting a reduced PTZ influence on astrocytes.

4.3. Comparison of the metabolic effects of ALCAR and valproate

PTZ induced many metabolic alterations which were not compensated for by either ALCAR or valproate. The latter did, however, increase the amount of glutathione. A major metabolic effect of valproate is potentiation of GABAerigc metabolism (Loscher, 1981, 1999, 2002) and ALCAR may be preferentially metabolized in GABAerig neurons (Scafidi et al., 2010). PTZ, on the other hand, diminishes GABAergic inhibition through binding to the GABA-A receptor. In spite of these similarities, the metabolic changes caused by valproate were not paralleled by the changes induced by ALCAR supplementation.

4.4. The effects of ALCAR and valproate on monoamine metabolism in PTZ kindling

It has been shown that monoamine neurotransmission and metabolism is of importance in seizure control in a number of animal models including the PTZ kindling model (Szyndler et al., 2010). There are many studies supporting the hypothesis that dopamine exerts an antiepileptic effect in man and animal models (Starr, 1996). In dopamine D2 receptor knock-out mice, systemic administration of the glutamatergic agonist kainic acid caused substantial hippocampal cell death compared to wild type (Bozzi et al., 2000). The results suggest a neuroprotective role of dopamine against excitotoxicity. Interestingly, dopamine concentration was increased in the HF of PTZ-kindled mice in the present study. This is in agreement with results reported from the HF of rat pups (age P10, P14, P17, P21) subjected to 60 min of PTZ induced status epilepticus (el Hamdi et al., 1992). However, Szyndler et al. (2010) did not obtain an increase in dopamine concentration in hippocampus of PTZ-kindled animals compared to chronic saline-injected animals. But there was a significant increase in dopamine in PTZ-kindled animals compared to single saline-injected animals. These findings suggest that dopamine alterations may be a result of stress caused by chronic injections per se. However, in our study, all animals had the same number of i.p. injections.

Supplementation with ALCAR attenuated the increase in dopamine in HF in PTZ-kindled mice. The fact that PTZ-kindled mice had a significantly higher level of dopamine compared to control mice, but PTZ–ALCAR mice did not, may indicate that ALCAR deprived the brain of a protective measure. However, the same result may indicate that ALCAR prevented the underlying cause of dopamine increase, possibly hippocampal cell death. Even though NAA was not reduced, such a measurement may not be sensitive enough to detect small alterations in subpopulations of hippocampal neurons. Previously, we have shown that ALCAR supplementation in healthy mice increased noradrenaline content in the HF and that of serotonin in the cortex compared to animals receiving saline injections, but no effects on dopamine were found (Smeland et al., in press). Interestingly, the present data demonstrate that ALCAR did not increase the amounts of serotonin and noradrenaline, but did affect dopamine levels in PTZ kindled animals. This indicates that ALCAR has different effects on monoamine levels in healthy control mice compared to those in PTZ subjected mice.

5. Conclusion

ALCAR was able to attenuate the PTZ-induced reduction in ^{13}C labeled alanine in the hippocampal formation. It also modified the dopamine increase in the HF. Thus, even though ALCAR did not delay the kindling process, chronic supplementation did normalize some metabolic alterations in the PTZ-kindled brain. However, it remains unanswered if these alterations could improve current epilepsy treatment. The fact that valproate failed to properly reduce kindling development in the present study, argues that ALCAR may have antiepileptic effects that the present investigation failed to demonstrate. In light of our findings, the possibility of ALCAR as a supplement to current antiepileptic treatment is worthy of further investigation.

Conflict of interests

The authors declare no conflict of interest.

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Paper III

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