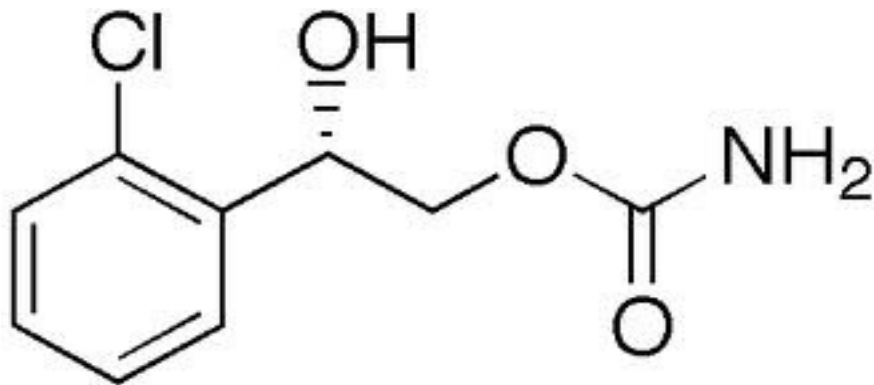


The effects of carisbamate on [1-¹³C]glucose and [1,2-¹³C]acetate metabolism in the limbic structures of the lithium-pilocarpine model of temporal lobe epilepsy in rats



Master's thesis in Neuroscience

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Abbreviations

Acetyl CoA	acetyl coenzyme A
ADP	adenosine diphosphate
ALE	absence like epilepsy
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CIT	citrate
CNS	central nervous system
Creatine Pho.	creatine phosphate
CRS	carisbamate
CRS-ALE	carisbamate treated absence like epileptic rats
CRS-TLE	carisbamate treated temporal lobe epileptic rats
GABA	gamma aminobutyric acid
GABA T	GABA transaminase
GAD	glutamic acid decarboxylase
Gln	glutamine
Glu	glutamate
GS	glutamine synthetase
HPLC	high performance liquid chromatography
i.p.	intraperitoneal
IBE	International Bureau for Epilepsy
ILAE	International League Against Epilepsy
MAL	malate
NAA	N-acetyl aspartate
NAD ⁺ /NADH	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
PAG	phosphate activated glutaminase
PC	pyruvate carboxylase
PDH	pyruvate dehydrogenase
PLC-DG-IP3	phospholipase C-diacylglycerol-inositol triphosphate
PPP	pentose phosphate pathway
PTZ	pentylentetrazol
PYR	pyruvate
ROS	reactive oxygen species
s.c.	subcutaneous
SE	status epilepticus
SEM	standard error of the mean
TCA	tricarboxylic acid
TLE	temporal lobe epilepsy
TSP	trimethylsilyl propanoic acid
α -ABA	amino butyric acid

Abstract

TLE is a type of focal epilepsy which is often resistant to medication. There is a need for the development of new drugs to treat patients and, even more important, to prevent epileptogenesis. Carisbamate (CRS) showed strong neuroprotective and antiepileptogenic properties in animal models. However, its mechanism of action is not yet clear. The aim of this project was to study neuronal and astrocytic metabolism in the limbic structures of rats using the lithium-pilocarpine model of TLE and to investigate the effects of CRS treatment on lithium-pilocarpine epileptic rats.

Status epilepticus (SE) was induced in rats by i.p. injection of lithium chloride followed by pilocarpine (25 mg/kg) 20 hrs later. CRS (90 mg/kg) was injected at 1 and 8 hr following SE and injections were repeated twice daily for 6 additional days. Another group received low doses of diazepam at 1 and 8 hrs after onset of SE and vehicle for 6 additional days. Lithium chloride and vehicle instead of pilocarpine and CRS were given to the control group. Two months after SE, animals were injected with [1-¹³C]glucose and [1,2-¹³C]acetate i.p. Twenty minutes later the rats were subjected to microwave fixation. The brains were removed and the limbic structures (amygdala, piriform/entorhinal cortex) were dissected. The extracts from the limbic structures were analyzed using HPLC, ¹H and ¹³C NMR spectroscopy.

Amounts of glutamate, aspartate, NAA, glutathione and taurine were reduced in the epileptic rats that didn't receive CRS. Decreased labeling of glutamate and glutamine from [1-¹³C]glucose was found. The transfer of glutamate from neurons to astrocytes was also impaired. These results show neuronal loss and/or disturbed mitochondrial neuronal metabolism. However, CRS treatment increased the amounts of glutamate, NAA, glutathione and taurine. CRS protected neurons and improved mitochondrial neuronal metabolism. The ¹³C labeling of [4,5-¹³C]glutamate/glutamine and [1,2-¹³C]GABA from [1,2-¹³C]acetate was reduced in the untreated rats. These results show reduced astrocytic metabolism in the untreated epileptic rats. CRS treatment resulted in large amounts of glutamine, [4,5-¹³C]glutamine and NAA in the ALE rats compared to the TLE group. In conclusion, lithium-pilocarpine caused reduced neuronal and astrocytic metabolism in the limbic structures of rats and CRS prevented a deterioration of neuronal metabolism while its effect on astrocytic metabolism was minimal.

1. Introduction

1.1. Brain cells

The brain contains two kinds of specialized cells for signaling: neurons and glia. The glial cells consist of astrocytes, oligodendrocytes, and microglia. Oligodendrocytes in the CNS and Schwann cells in the periphery produce myelin which insulates nerve cell axons. Microglia are phagocytic cells which remove debris after neuronal injury or death (Kandel *et al.* 2000). In the human cortex, there are about 1.4 astrocytes for each neuron (Nedergaard *et al.* 2003). Neurons and astrocytes are the two major types of cells in the brain cortex and the other cells account only for a small fraction of the volume (Hertz & Dienel 2002). Neurons are the main signaling units of the nervous system. They transmit information through electrical and chemical signals (Kandel *et al.* 2000).

Astrocytes are supporting cells with a variety of brain functions. They are important in providing energy, nutrients, neurotransmitter precursors to neurons and maintaining the right potassium concentration in the extracellular space. They also take up neurotransmitters and regulate synaptic activity. In addition, astrocytes have several proteins which can be used in neutralizing free radicals which are in the form of reactive oxygen species (ROS). They form end feet on the surface of blood vessels and may regulate cerebral blood flow. They are the most metabolically active glial cells in the brain (Ricci *et al.* 2009, Zwingmann & Leibfritz 2007).

1.2. Brain Metabolism

Although the human brain represents only 2% of the total body weight, it accounts for approximately 20% of the body's resting metabolism. About 75% of the energy consumption is used for signaling purposes of which maintenance of action potential is a major one. The rest is used for other essential cellular activities (Attwell & Laughlin 2001). Under physiological conditions, glucose is the main energy substrate for the adult brain (Zwingmann & Leibfritz 2003). Glucose is taken up from the blood circulation and transported through the blood brain barrier into the brain by specific glucose transporters. Then, glucose may enter into both neurons and astrocytes (Nehlig *et al.* 2004). At rest, both neurons and astrocytes take up glucose at the same rate, however when there is intense neuronal activation the rate of glucose uptake in

astrocytes increases while in neurons is unaltered (Chuquet *et al.* 2010). Approximately 66% of available glucose at the acetyl CoA stage in the adult brain is metabolized by neurons while 34% is metabolized by astrocytes (Qu *et al.* 2000).

1.2.1. Metabolism of Glucose

Glucose, in addition to its use as energy substrate, has several metabolic fates in the brain. It is used as a substrate for inositol biosynthesis and formation of glycogen. The carbon skeleton from glucose is also incorporated into several neurotransmitters, amino acids and other substrates including acetylcholine, lactate, glutamate, glutamine, aspartate, GABA, alanine, serine, and glycine (McKenna *et al.* 2012).

After entry, glucose is phosphorylated to glucose-6-phosphate in the cytosol. Depending on the energy status and type of cell, glucose-6-phosphate is either further catabolized via glycolysis, or goes through the pentose phosphate pathway (PPP) or is used in the generation of glycogen in astrocytes. The amount of glucose metabolized through glycolysis is higher than that entering the PPP (Kreft *et al.* 2012).

Glucose produces energy via Glycolysis and the tricarboxylic acid (TCA) cycle (Fig. 1). During glycolysis which occurs in the cytosol, glucose is metabolized into two molecules of pyruvate with a concomitant production of two molecules of ATP (McKenna *et al.* 2012). Pyruvate can also be metabolized into lactate by lactate dehydrogenase (LDH) or into alanine by alanine aminotransferase. In Mitochondria, pyruvate can either be decarboxylated into acetyl CoA by pyruvate dehydrogenase (PDH) (in both neurons and astrocytes) which finally enters into the TCA cycle or it can be carboxylated to oxaloacetate by pyruvate carboxylase (PC) (Hertz & Dienel 2002, McKenna *et al.* 2012). PC is found only in astrocytes (Yu *et al.* 1983). It is an anaploretic enzyme which is used to replenish TCA cycle intermediates or for adding net carbons to the TCA cycle following removal of carbon backbone for synthesis of glutamate and GABA (Sonnewald & Rae 2010). TCA cycle intermediates are lost by neurons during glutamatergic and GABAergic neurotransmission. Glutamate and GABA are made from α -ketoglutarate and removed from the synaptic cleft by astrocytes and neurons (McKenna *et al.* 2012).

Most of the ATP from glucose metabolism is obtained from the TCA cycle. Oxidative metabolism of glucose occurs in the mitochondria after PDH mediated conversion of pyruvate to acetyl CoA, which is oxidized to CO₂ and H₂O within mitochondria. Oxaloacetate condenses with acetyl CoA to form Citrate. An isoform of citrate is then decarboxylated resulting in formation of α -ketoglutarate which is also decarboxylated to succinate via succinyl CoA. Subsequent enzymatic reactions then change succinate into fumarate, malate and then finally to oxaloacetate (Hertz & Dienel 2002, McKenna *et al.* 2012).

The TCA cycle in addition to providing ATP, produces carbon skeletons for the synthesis of neurotransmitters and aminoacids. Glutamate, glutamine and GABA are obtained from α -ketoglutarate (McKenna *et al.* 2012).

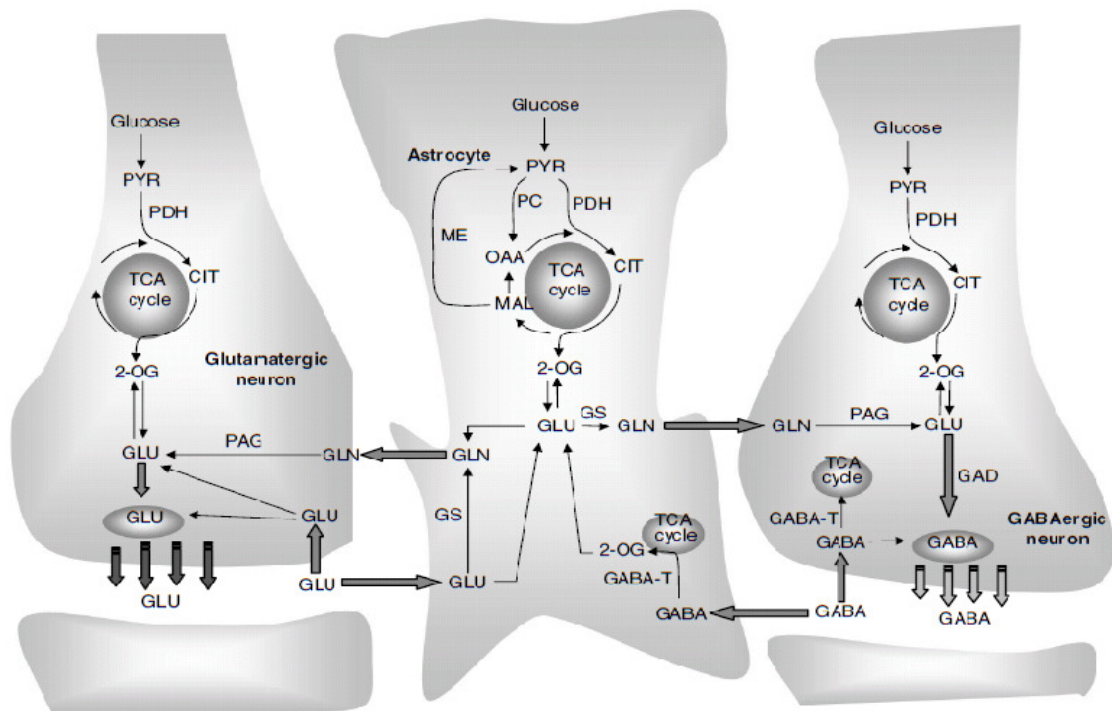


Figure 1: Through glycolysis, glucose is converted into pyruvate. By the action of PDH enzyme it can be converted into acetyl CoA in both neurons and astrocytes and enters into the TCA cycle. In astrocytes, pyruvate can be converted into oxaloacetate by PC. Acetyl CoA condenses with oxaloacetate and then after subsequent enzymatic reactions forms α -ketoglutarate (2-oxo glutarate) (2-OG). α -ketoglutarate can be converted into glutamate and then into GABA (in GABAergic neurons). The figure is taken from (Sonnewald 2005). Abbreviations: CIT, citrate; GAD, glutamate decarboxylase; GABA-T, GABA transaminase; GLN, glutamine; GLU, glutamate; GS, glutamine synthetase; MAL, malate; ME, malic enzyme; 2-OG, 2-oxoglutarate (α -ketoglutarate); PAG, phosphate-activated glutaminase; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PYR, pyruvate; TCA, tricarboxylic acid.

1.2.2. Metabolism of Acetate

Acetate is preferentially metabolized in astrocytes than in neurons (Cerdan *et al.* 1990, Waniewski & Martin 1998) because of its rapid uptake by monocarboxylate transporters. Acetate is converted into acetyl-CoA by acetyl-CoA synthetase which can enter into the TCA cycle by condensing with oxaloacetate (Waniewski & Martin 1998).

1.3. Glutamate-glutamine cycle

Glutamate is released by neurons into the extracellular space. Then the astrocytes take up glutamate (Danbolt 2001) and convert it to glutamine by glutamine synthetase (GS) which is astrocyte-specific (Norenberg & Martinez-Hernandez 1979). This glutamine is released from the astrocytes into the extracellular space from where it can be taken up by neurons and transformed back to glutamate by phosphate activated glutaminase (PAG). This trafficking of glutamate and glutamine between neurons and astrocytes is known as the glutamate-glutamine cycle (reviewed in (Bak *et al.* 2006)). The glutamate-glutamine cycle is shown in Figure 2.

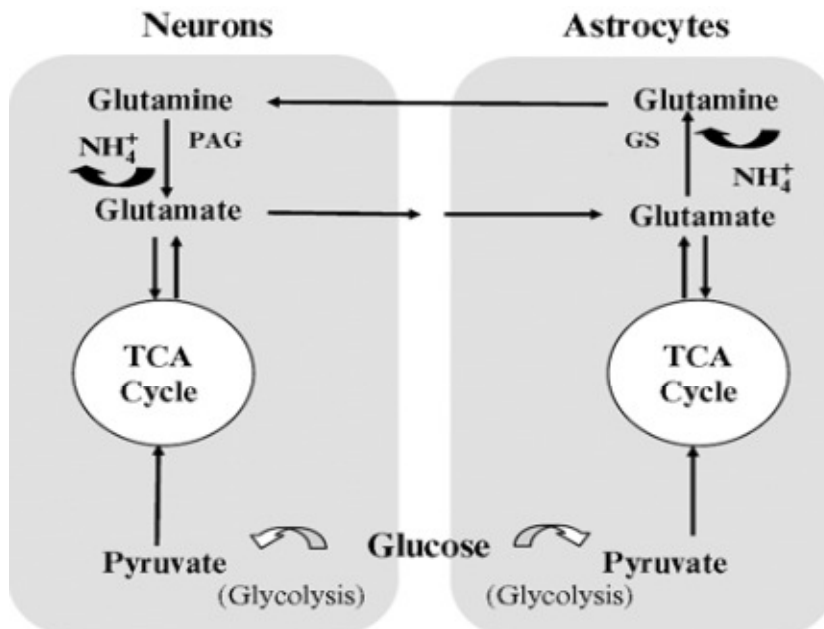


Figure 2: The glutamate-glutamine cycle. Glucose is metabolized into pyruvate. Pyruvate enters into the TCA cycle of both neurons and astrocytes and synthesizes glutamate through α -ketoglutarate. Glutamate is released from neurons and taken up by astrocytes. Astrocytes convert glutamate into glutamine using GS and glutamine is then taken to neurons and is converted back to glutamate by PAG. The figure is taken from (Zwingmann & Leibfritz 2007). Abbreviations: GS, glutamine synthetase; PAG, phosphate-activated glutaminase; TCA: tricarboxylic acid.

1.4. Epilepsy

Epilepsy is a common neurological disorder that affects 1 to 3% of the population (Shneker & Fountain 2003). According to the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE), *'An epileptic seizure is a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain. Epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure'* (Fisher *et al.* 2005).

The revised ILAE classification categorizes seizures into generalized, focal and unknown type. Generalized epileptic seizures are seizures 'originating at some point within, and rapidly engaging, bilaterally distributed networks' and are classified into tonic-clonic, absence, myoclonic, tonic, clonic and atonic. However, focal epileptic seizures are seizures 'originating within networks limited to one hemisphere' and are divided according to those 'without impairment of consciousness or awareness' and 'with impairment of consciousness or awareness' (Berg *et al.* 2010).

1.4.1. Temporal lobe epilepsy (TLE)

Temporal lobe epilepsy (TLE) is a type of focal epilepsy. Focal epilepsies are typical for 60% of all patients with epilepsy and TLE is the most common type of focal epilepsy. It is often resistant to antiepileptic drugs and often referred for surgery (Tellez-Zenteno & Hernandez-Ronquillo 2012). TLE includes those seizures that arise in the temporal lobe (Bertram 2009).

There are two kinds of TLE: medial TLE and neocortical or lateral TLE. The medial TLE is the major type of TLE and it involves those structures found on the medial side of the temporal lobe. Lateral TLE includes the other structures outside the medial temporal lobe. The medial structures which are important in the generation of spontaneous seizures include hippocampus, amygdala, thalamus and parahippocampal gyrus. Hippocampal sclerosis is the most common histopathological finding in medial TLE (Bertram 2009). Medial TLE is characterized by alterations in the hippocampus

including hardening, a reduction in size and neurodegeneration (O'Dell *et al.* 2012).

1.4.2. Epileptogenesis

It is a process by which the brain undergoes cellular, molecular and neuronal network changes after damage in the brain which eventually leads to hyperexcitability of neuronal network and development of spontaneous seizures which are severe and more frequent. An initial brain insult leads to acute seizure activity. The common initial insults include infection, traumatic brain injury, stroke, febrile seizures and status epilepticus (SE) (Pitkanen *et al.* 2007). This is followed by a silent/latent period when a lot of those brain alterations occur. After this period, spontaneous seizures emerge. The silent period lasts months to years in humans and 2-12 months in most rodent animal models. Neuronal degeneration, gliosis, mossy fiber sprouting and network reorganization are some of the changes that occur in the latent period (Rakhade & Jensen 2009). Figure 3 shows the development of epileptogenesis in patients with epilepsy and experimental models of epilepsy.

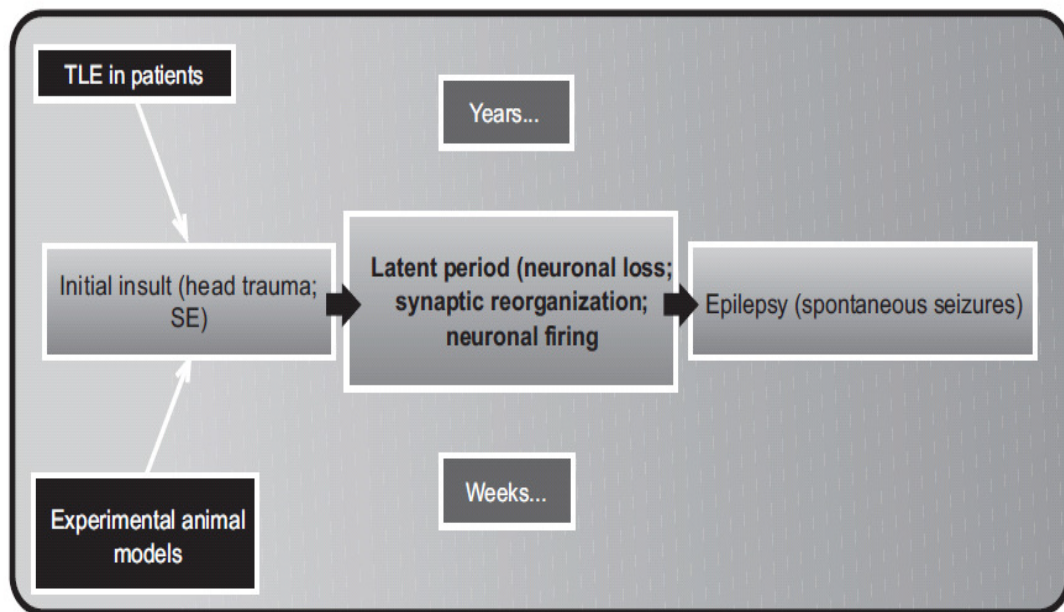


Figure 3: Schematic diagram of epileptogenesis in patients and experimental models (the figure is taken from Ferandes *et al.* 2010). An initial brain damaging insult leads to epileptogenesis that eventually leads to development of spontaneous seizures. Abbreviation: SE, status epilepticus; TLE, temporal lobe epilepsy

1.4.3. Limbic structures in epilepsy

The hippocampus, amygdala, and the surrounding cortex, including the entorhinal, perirhinal, and parahippocampal cortices constitute the medial temporal lobe (Kiernan 2012). TLE is mostly associated with hippocampal sclerosis, however temporal lobe areas other than the hippocampus including the entorhinal cortex, amygdala and limbic thalamus are also often injured in various animal models of epilepsy. The volume of these structures was reduced following TLE using MR volume measurements (Jutila *et al.* 2001).

Neuronal loss has been found in entorhinal and piriform cortex in rat models of TLE (Du *et al.* 1995). The amygdala is also one component of the temporal lobe that is damaged in TLE. Neuronal loss in amygdala is shown by significant volume reduction in imaging studies. The density of GABAergic inhibitory neurons is also reduced. The loss of inhibitory neurons is thought to be the reason for lowering of seizure threshold in this region (Pitkanen *et al.* 1998). In the lithium pilocarpine model of TLE, extensive neuronal loss was observed in amygdala and the piriform and entorhinal cortex in rats (Roch *et al.* 2002).

1.4.4. Brain metabolic disturbances in epilepsy

Epilepsy is characterized by an imbalance between excitatory and inhibitory neurotransmission in the brain mainly due to disturbances in neurotransmitter metabolism (Sonnewald & Kondziella 2003). Particularly, there is disturbed glutamate and glucose metabolism and astrocytic alterations in both human and animal models of epilepsy (Pfund *et al.* 2000, Melo *et al.* 2005). Studies have shown metabolic changes during the latent and chronic periods of epilepsy. In the lithium-pilocarpine model of epilepsy in rats, neuronal metabolism was found to be altered while astrocytic metabolism was unchanged in hippocampal formation, cerebral cortex and cerebellum (Melo *et al.* 2005). Altered neuronal metabolism and neuronal-glia interactions were observed in limbic structures in the chronic phase of the kainate model of medial TLE (Alvestad *et al.* 2008). In the latent phase of kainite induced TLE, decreased astrocytic mitochondrial metabolism and disturbed neuronal-glia interactions were observed (Alvestad *et al.* 2011). Energy metabolism and the glutamate-glutamine cycle are impaired in epilepsy (Petroff *et al.* 2002a).

1.5. Carisbamate (CRS)

CRS ((S)-2-O-carbamoyl-1-O-chlorophenyl-ethanol); Fig. 4) is a novel neuromodulator which was under development for the treatment of epilepsy. It was initially developed by SK biopharmaceuticals (Fairlawn, New Jersey) and was later under development by Johnson and Johnson Pharmaceutical Research and Development (La Jolla, California) (Novak *et al.* 2007).

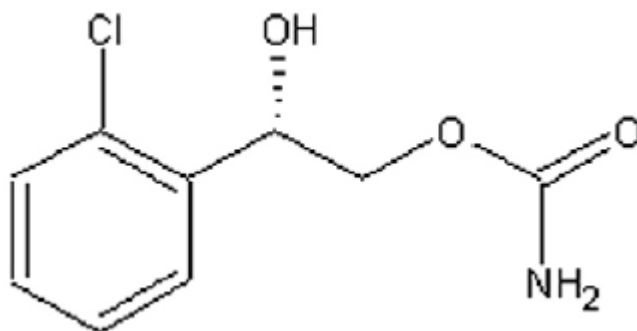


Figure 4. Chemical structure of carisbamate

It is a monocarbamate with similar structure to the antiepileptic drug felbamate. Felbamate is a broadspectrum antiepileptic drug. It blocks NMDA receptors and also inhibits voltage gated sodium and calcium channels. However, it has serious adverse effects including aplastic anemia and liver failure as a result of production of a toxic reactive metabolite (felbamate 2-phenylpropanal (ATPAL)). CRS however, does not produce the reactive metabolite and as a result is free of the side effects associated with felbamate (Landmark & Johannessen 2008).

CRS has broad spectrum anticonvulsant activity in rodent models of generalized and partial seizures. Its effectiveness was confirmed in maximal electroshock seizure, pentylenetetrazole (PTZ), bicuculline, picrotoxin and audiogenic seizure tests in mice when it is administered intraperitoneally and in lamotrigine resistant amygdale kindled and 6HZ model in rats. It prevented and interrupted lithium-pilocarpine-induced status epilepticus (White *et al.* 2006).

CRS was also effective in the Genetic Absence Epilepsy Rat from Strasbourg (GAERS) and in the genetic model of sound-induced convulsive seizures, the

audiogenic Wistar AS rat. It has shown broad efficacy on primary generalized seizures of both the tonic reflex and the absence type in genetic models of epilepsy (Francois *et al.* 2008, Nehlig *et al.* 2005). It also displayed neuroprotective properties in the CA1 area of the hippocampus, the thalamus, amygdala and ventral cortices (Francois *et al.* 2005).

CRS reduced the frequency of spontaneous motor seizures in rats with kainate-induced epilepsy (Grabenstatter & Dudek 2008). It also prevented the development and generation of epileptiform discharges when administered following SE-like injury *in vitro* in cultured hippocampal neurons (Deshpande *et al.* 2008).

The mechanism of action of CRS is not yet clear however, CRS selectively depressed excitatory neurotransmission in piriform cortical layer Ia-afferent terminals by blocking Na⁺ channels and increasing Cl⁻ conductance presynaptically (Whalley *et al.* 2009). It also caused a concentration-, voltage- and use-dependent inhibition of rat Nav1.2. In rat hippocampal neurons, CRS similarly blocked voltage-gated sodium channels, and inhibited repetitive firing of action potentials in a concentration-dependent manner (Liu *et al.* 2009). CRS reduced glutamatergic transmission by an action potential-dependent presynaptic mechanism and consequently inhibited excitatory synaptic strength in the dentate gyrus without affecting GABAergic transmission (Lee *et al.* 2011).

In a study done in lithium-pilocarpine epileptic rats, treatment with CRS showed strong neuroprotective potential of hippocampus, ventral cortices, thalamus, and amygdala. It also prevented mossy fiber sprouting in the dentate gyrus of the hippocampus and delayed or suppressed the occurrence of spontaneous motor seizures. The most fascinating result of this study was that administration of CRS led to two different pathological outcomes. One is the classical pharmacoresistant TLE and the other is ALE. CRS was able to transform pharmacoresistant TLE into a milder and treatment responsive ALE. The molecular mechanism of its action is not yet clear (Francois *et al.* 2011).

In spite of very promising preclinical studies using animal models, clinical trials showed CRS had limited efficacy for most pharmacoresistant patients (Eastman *et al.*

2011). Because of inconsistent antiepileptic efficacies in phase III clinical trials, the application for a marketing authorization for use in the treatment of partial-onset seizures in patients with epilepsy was withdrawn by the company (Sperling *et al.* 2010, Faught *et al.* 2008, Halford *et al.* 2011).

On the other hand, CRS is the first available molecule that displayed so strong and extended neuroprotective and disease-modifying (antiepileptogenic) properties in rodents. Moreover, the change in chronic seizure expression is accompanied by complete behavioral and cognitive recovery. Therefore, CRS still remains an interesting molecule to study as a neuroprotective and antiepileptogenic agent given its structural similarities with an antiepileptic drug felbamate and its mechanism of action (Francois *et al.* 2011).

1.6. Theoretical background of the methods

1.6.1. Lithium-Pilocarpine model of TLE

The Pilocarpine model is a model of epilepsy which reproduces the major features of human TLE in rats or mice. The main features of TLE include epileptic foci in the limbic system, an acute period of limbic SE, a silent (latent) period, a chronic period of spontaneous recurrent seizures (SRSs) and the presence of hippocampal sclerosis (Cavalheiro 1995). Systemic administration of pilocarpine in rats produces epileptiform activity in the limbic structures accompanied by motor limbic seizures, limbic SE and widespread brain damage (Turski *et al.* 1983). The induction of SE by pilocarpine leads to significant and widespread neuronal death in several brain areas. The brain damage usually occurs in the hippocampus, the piriform and entorhinal cortices, the amygdala and the thalamus (Andre *et al.* 2007, Scorza *et al.* 2009).

Pilocarpine can be combined with other drugs such as lithium and picrotoxin depending on the desired characteristics of the model. The most commonly used model is the lithium-pilocarpine combination. Administration of lithium is generally done 24 hours before the induction of SE. One of the advantages of combining lithium with pilocarpine is to reduce the dose of pilocarpine used to induce seizures and thereby reduce mortality. In addition, there is reduced variability to onset of SE. Lithium pretreated rats are also highly sensitive to the effect of pilocarpine and there is a 100% success rate in the generation of seizures (Clifford *et al.* 1987, Curia *et al.* 2008).

Muscarinic activation is thought to be the mechanism for induction and propagation of seizures. This was shown by systemic administration of atropine which is a muscarinic antagonist which blocked seizures. However, once the seizure is induced muscarinic receptors are less important and the spread of seizure activity is related to other, non cholinergic, pathways especially the glutamatergic pathway. Glutamate receptors are activated and this was evidenced by damage to those brain areas with higher density of glutamate receptors including hippocampus and several thalamic and amygdaloid nuclei (Clifford *et al.* 1987).

Lithium potentiates the activity of pilocarpine by several mechanisms. It is a proconvulsant and also reduces noradrenaline activity. It also increased release of acetylcholine and caused other alterations in second messenger systems (Clifford *et al.* 1987). Activation of M1 muscarinic receptors activates the PLC-DG-IP3 pathways which results in Ca^{2+} and K^+ currents and increases brain excitability. High concentrations of calcium in turn produce large release of glutamate which induces SE. Glutamate, by activating its receptors, will allow more entrance of Ca^{2+} into postsynaptic cells thereby causing excitotoxicity and cell death (Scorza *et al.* 2009).

1.6.2. High Performance Liquid Chromatography (HPLC)

Chromatography is a powerful separation method which allows separation, identification and determination of closely related substances from complex mixtures. A sample is dissolved in a mobile phase and the mobile phase is forced to pass through a stationary phase which is fixed in a column or a solid surface. The two phases have characteristics that allow the components of the sample to distribute themselves between the mobile and the stationary phases to different degrees. Therefore, when the mobile phase flows, those components that are strongly retained in the stationary phase will elute last and those components that are retained weakly will elute first. Depending on their difference in migration rate or retention time, each components will separate in to discrete bands or zones along the length of the column Upon elution the bands will then be detected by a detector and quantified (Skoog *et al.* 2007).

In high performance liquid chromatography (HPLC), the mobile phase is liquid and the stationary phase uses small particle columns. The sample is injected into the path

of the mobile phase and it is pumped through the column using high pressure. The eluted substances produce peaks, the areas under which are proportional to the concentration of each constituent of the sample and are identified based on their retention time by comparing with a standard run under identical conditions. The peak data with standard calibration is used for quantification of sample components (Nair *et al.* 2004, Dong 2006, McMaster 2007).

For quantification purposes, standards with known composition and concentration are used and their peak areas are plotted against concentration. The plot yields a calibration curve which is used for analysis (Skoog *et al.* 2007). HPLC is a valuable tool for analysis of many important biological metabolites.

1.6.3. Nuclear Magnetic Resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy methods are very powerful and versatile tools for analyzing biochemical samples. The methods are non-destructive and non-invasive. In addition, it is possible to make repetitive measurements (Kiinnecke 1995).

NMR spectroscopy is a method which uses the magnetic properties of certain atomic nuclei (^1H , ^{13}C , ^{15}N , ^{31}P) (Scott & Baxter 1981). Some atomic nuclei have a small magnetic field because of the nucleus with charge and spinning property. When these nuclei are immersed in a strong uniform magnetic field (B_0), the motion of each nucleus changes and the nuclei starts to precess, meaning the nucleus which was already rotating with its axis of rotation also rotates with the direction of the magnetic field (Berry & Bulpitt 2008).

The precession occurs with a Larmor frequency (f_0) (the rate of precession of a spin) which depends on the nuclei's gyromagnetic ratio (γ) and the external magnetic field (B_0):

$$f_0 = \gamma * B_0$$

The gyromagnetic ratio is constant for a particular nucleus. The difference in the gyromagnetic ratio for nuclei of different atoms means each nuclei will have a characteristic Larmor frequency to precess in the presence of a strong magnetic field.

The precessional motion can take up two preferred states in which the small magnetic fields align parallel (spin-up) or antiparallel (spin-down) to the direction of the external magnetic field (B_0). When an external magnetic field is applied, more spins exist in the spin-up state (Berry & Bulpitt 2008).

During the NMR experiment, an external radiofrequency pulse is supplied at a Larmor frequency of a particular nucleus. Energy will get absorbed and those spins in the up (low energy) state will move to the spin-down (high energy) state. This process is called resonance (Balci 2005). The signal obtained in the NMR spectrum is due to the energy differences in populations between nuclei in the high energy state and the lower energy state. When radiofrequency is turned off, the sample regains equilibrium; nuclei relax back to the ground state and release energy which leads to detection of NMR signal. The time spent to relax the nuclei back (relaxation time) determines the width of the peaks. The magnitude of the NMR signal is dependent on the nuclei's gyromagnetic ratio and the strength of the outer magnetic field (Berry & Bulpitt 2008, Balci 2005). Each magnetic nucleus in a molecule experiences a different chemical environment that can affect the Larmor frequency. Electrons surrounding a nucleus create a local magnetic field which opposes the influence of the external magnetic field. This phenomenon is called shielding and causes the chemical shift. The chemical shift is the difference between the resonance frequency of the nuclei and a standard (Balci 2005).

1.6.3.1. ^{13}C NMR spectroscopy

^{13}C is a naturally occurring carbon isotope with a nuclear magnetic moment and 1.1% natural abundance but it has a very low sensitivity. However, ^{13}C enriched substances can be used to study brain metabolism (Sonnewald *et al.* 2011). ^{13}C labeling derived from labeled precursors at specific carbon positions of small metabolites, TCA cycle intermediates and amino acids will be detected using ^{13}C NMR spectroscopy. The position of the label depends on the metabolic pathways of the labeled carbon molecule. Hence, from the labeling pattern it is possible to study metabolic pathways (Hassel *et al.* 1995). The fact that neurons and astrocytes have different enzymes and transporters is the basis for metabolic compartmentation which can be studied using NMR spectroscopy (Melo *et al.* 2005).

1.6.3.2. ^1H NMR spectroscopy

^1H NMR is the most widely used NMR spectroscopy technique for the brain as a result of its higher sensitivity as compared to other nuclei (Ross & Bluml 2001). ^1H NMR spectroscopy is able to detect a number of metabolites that offer a biochemical insight into the underlying metabolic basis for many disorders in the brain (Rosen & Lenkinski 2007).

2. Aims of the thesis

About one third of patients with epilepsy have poor control of seizures and the seizures are resistant to medication. Moreover, many antiepileptic drugs are often taken chronically and are associated with marked adverse effects (Engel & Schwartzkroin 2005). Those drugs that are currently available in the pharmacological treatment of epilepsy are intended to suppress seizures and they have no antiepileptogenic or disease modifying property. The antiepileptic drugs are thus not able to prevent the development of chronic epilepsy. There is therefore a need for therapeutic targets that can be used in the prevention of epileptogenesis (Rakhade & Jensen 2009, Pitkanen 2010).

CRS is the first experimental drug that showed antiepileptogenic properties in animal models. It also displayed strong neuroprotective activity. However, the mechanism of action of CRS is not clearly known (Francois *et al.* 2011). Therefore, the present project is aimed at clarification of possible mechanisms of CRS's action and the potential role of CRS in the inhibition-excitation balance in brain tissue of rats with different types of epilepsy. We investigated the effects of CRS administration on brain metabolism in the lithium-pilocarpine animal model of TLE.

Our aim was to address the following questions:

How are the limbic structures affected by lithium-pilocarpine administration to rats?

How are neuronal-glia interactions affected in limbic structures of the lithium-pilocarpine model of TLE?

Can treatment with CRS alter the damage in the limbic structures caused by seizures induced by lithium-pilocarpine administration?

Does CRS affect brain metabolism?

Are there metabolic differences between the CRS treated CRS-TLE and CRS-ALE groups?

3. Materials and Methods

3.1. Experimental procedures

All the animal experimentation was done by Prof. Astrid Nehlig in Strasbourg, France. The tissue extraction and analysis using HPLC and ^1H and ^{13}C NMR spectroscopy was done at NTNU. I didn't participate in the animal experiments for the rats but I have taken part in similar experiments and I am familiar with the procedures.

3.1.1. Animals

Adult male Sprague-Dawley rats were provided by Charles River (L'Arbresle, France). All animals were maintained under controlled conditions at 20-22°C (light/dark cycle, 7.00 a.m.-7.00 p.m. lights on), with free access to food and water. The animal experiments were done in accordance with the rules of the European Communities Council Directive of November 24, 1986 (86/609/EEC), the UK Animals (Scientific procedure) Act, 1986 and the French Department of Agriculture (License N° 67-97). The ethical Animal Research Committee Board of Louis Pasteur University agreed with the protocol (CREMEAS #AL/01/04/03/07) and all efforts were made to reduce the number of animals used and to minimize animal suffering.

3.1.2. Induction of status epilepticus and carisbamate treatment

All rats were injected with Lithium chloride (3 meq/kg, i.p., Sigma, St Louis, Mo, U.S.A.) (to enhance the action of pilocarpine) intraperitoneally (i.p.) and about 20 h later, received also methylscopolamine bromide (1 mg/kg, Sigma) subcutaneously (s.c.) to limit the peripheral effects of the convulsant. 30 min after methylscopolamine, SE was induced by s.c. injection of pilocarpine hydrochloride (25 mg/kg, Sigma) and its onset was determined based on behavioural observation. SE was considered as starting when rats did not react to contact and experienced hypersalivation, which occurred usually after 3-4 stage IV-V seizures.

Lithium chloride and vehicle instead of pilocarpine and CRS were given to the control group. Another group received two injections of diazepam (DZP, 2.5 and 1.25 mg/kg: Valium, Roche, France) an hour and 8 hours later respectively after lithium pilocarpine treatment on the day of SE and then vehicle for six days. This group didn't receive CRS. The purpose of administering low doses of diazepam is to reduce

mortality by producing myorelaxant and anxiolytic effects. It doesn't stop the seizures or SE. The treatment groups received carisbamate (90mg/kg; dissolved in 45% hydroxypropyl-beta-cyclodextrin (Acros Organics, Geel, Belgium)) intraperitoneally an hour after the onset of status epilepticus and a second injection 8 hours later. This dose was chosen because it displayed significant neuroprotective effect, low mortality and a significant disease-modifying effect (Francois *et al.* 2011). This procedure was done again for six more days. Carisbamate (90mg/kg, i.p) treatment produced two outcomes: one group with classical pharmacoresistant TLE associated with moderate neuroprotection and a second group with absence like epilepsy associated with extended neuroprotection and responding to classical treatments (for details see (Francois *et al.* 2011)). In our experimental set up, we therefore had four groups: normal control group that received vehicle, untreated lithium-pilocarpine TLE group (UT-TLE) that received diazepam, CRS treated lithium pilocarpine TLE group (CRS-TLE) and CRS treated lithium pilocarpine ALE group (CRS-ALE).

3.1.3. Epileptogenesis

During the two months after SE, animals were daily video recorded for 10 h. Animals that displayed the first motor SRS were taken off the system and were kept until 2 months after SE to be used in the experiment. Rats that didn't exhibit motor SRS were sacrificed after two months of video recording. They were considered to develop ALE (Francois *et al.* 2011). Two rats in the UT-TLE group and 1 rat in the CRS treated groups died. The final number of rats used for this study was 7 control rats, 4 UT-TLE rats, 11 CRS-TLE rats and 7 CRS-ALE rats.

3.1.4. Injection of labeled glucose and acetate

Two months after SE, the epileptic fate of the animals was known. The animals were injected with [1-¹³C]glucose and [1,2-¹³C]acetate i.p. 20 min later the rats were subjected to microwave fixation followed by liquid nitrogen emersion of the heads. The brains were removed and the following areas were dissected: hippocampus, limbic structures (amygdala, piriform/entorhinal cortex) and thalamus and the brain tissue were stored at -80 °C till extraction. Four samples (one in the control, two in the CRS-TLE and one in the CRS-ALE) were discarded because of problem with injection and/or sacrifice. I worked only on the limbic structures (amygdala, piriform/entorhinal cortex) extracted from the brain tissue.

3.1.5. Tissue extraction

Methanol and α -ABA were added and tissue was homogenized using a Vibra Cell sonicator (Model VCX 750, Sonics & Materials, Newtown, CT, USA) followed by addition of water and chloroform. The samples were centrifuged at 3,000 \times g at 4⁰C for 15 min and the supernatants were transferred to a new tube (tube 2) and stored in ice. Methanol, chloroform and water were again added to the remaining tissue and centrifugation was done. The supernatants were again transferred to the second tube (tube 2). Methanol, water and chloroform were re-added and tissue was extracted. Supernatants were collected. Water was also added to the second tube and the samples were centrifuged in order to separate the lipids from the supernatant with intracellular extracts. Additional water and methanol were also added and supernatants were collected. Finally, the supernatants were lyophilized to remove water and methanol and re-suspended in D₂O before being lyophilized once more in order to minimize the proton content of water. The tissue samples from all control and treated groups were analyzed using ¹³C-NMR spectroscopy, ¹H-NMR spectroscopy and HPLC. The methanol-chloroform extraction procedure is attached as an appendix.

3.1.6. HPLC

Amino acids were studied using HPLC (1100 series; Agilent Technologies, Santa Clara, CA, USA) followed by fluorescence detection. A liquid mobile phase passes through a column containing a stationary phase. Ophthaldialdehyde was used as a derivatization agent for the amino acids. Amino acid components were separated on a reverse phase column, Zorbax SBC18 column (4.6 \times 150mm², 3.5-micron; Agilent Technologies) that is a non polar stationary phase in combination with a polar mobile phase. Gradient elution method, in which the mobile phase composition is changed, was used to elute mixture components from the stationary phase. To achieve optimal separation and faster elution of the nonpolar analytes, a gradient of two eluents were used: one of phosphate buffer (10 mM, pH 7.1) and the other of methanol. Standards with different concentrations were run with the samples. Relevant peaks from the HPLC spectra were identified and integrated. α -ABA was used as an internal standard for quantification. Amounts were corrected for the weight of the tissue.

3.1.7. ^1H and ^{13}C Nuclear Magnetic Resonance Spectroscopy

The amounts of isotopomers from metabolism of [$1\text{-}^{13}\text{C}$]glucose and [$1,2\text{-}^{13}\text{C}$]acetate and total amounts of selected metabolites were quantified using ^{13}C and ^1H NMR spectroscopy. Lyophilized brain tissue extracts were dissolved in 120 μl D_2O containing 99.0% (TSP) and 0.1% ethylene glycol as internal standards. The samples were transferred to SampleJet tubes ($3.0 \times 103.5\text{mm}^2$) for insertion into the SampleJet autosampler (Bruker BioSpin GmbH, Rheinstetten, Germany). All samples were analyzed using a QCI CryoProbe 600 MHz ultrashielded Plus magnet (Bruker BioSpin GmbH). ^1H NMR spectra were obtained using: pulse angle of 90° , acquisition time of 2.66 seconds and a relaxation delay of 10 seconds. The number of scans was 128. 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. The number of scans was typically 10,000.

3.1.7.1. Labeling patterns

By simultaneous injection of [$1\text{-}^{13}\text{C}$]glucose and [$1,2\text{-}^{13}\text{C}$]acetate and doing NMR spectroscopy analysis of brain extracts, it is possible to study and obtain information about astrocytic and neuronal interactions in the same animal. Injection of [$1\text{-}^{13}\text{C}$]glucose and [$1,2\text{-}^{13}\text{C}$]acetate results in the efficient labeling of many metabolites. Label from [$1\text{-}^{13}\text{C}$]glucose is quantified by analyzing the singlet peaks in different compounds while label from [$1,2\text{-}^{13}\text{C}$]acetate is quantified by analyzing doublets in the NMR spectrum which show predominantly astrocytic metabolism. Glutamine is labeled more from [$1,2\text{-}^{13}\text{C}$]acetate (doublet) than [$1\text{-}^{13}\text{C}$]glucose (singlet); for glutamate and GABA, the opposite is true. N-acetylaspartate (NAA) in the C-6 position, alanine, lactate and succinate are mainly labeled from glucose. Taurine, creatine, and myoinositol are not labelled and results in singlets (Sonnewald & Kondziella 2003). A typical ^1H and ^{13}C NMR spectrum of a tissue extract from the limbic structures of a rat, after the simultaneous injection of [$1\text{-}^{13}\text{C}$]glucose and [$1,2\text{-}^{13}\text{C}$]acetate, is shown in Figure 5 and Figure 6.

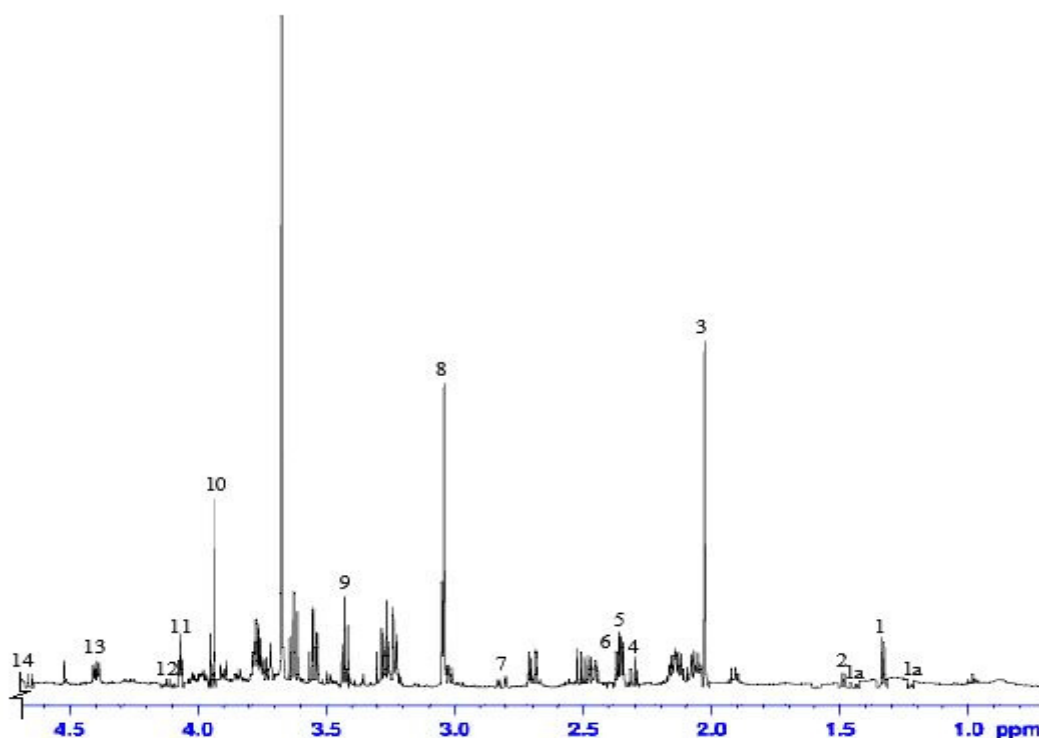
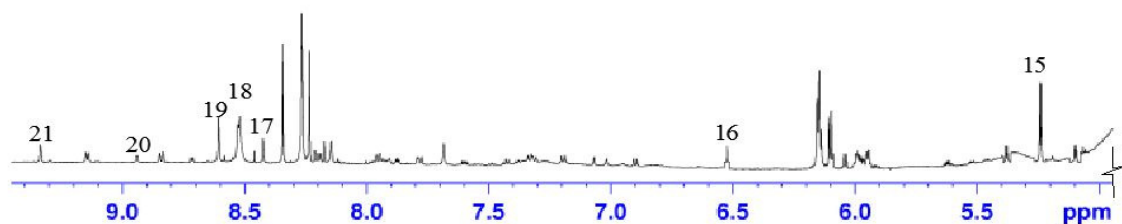


Figure 5: Typical ^1H NMR spectrum from limbic structures extract of rats. Rats were injected with $[1-^{13}\text{C}]$ glucose and $[1,2-^{13}\text{C}]$ acetate intraperitoneally and brains were microwave irradiated 15 minutes later and were dissected and extracted with methanol-chloroform extraction. The following peaks were identified and quantified: **1a**-lactate ^{13}C -3, (doublet) C-3, **1**- lactate C-3, (doublet) **2**- alanine C-3 (triplet), **3**- N-acetyl aspartate C-6 (singlet), **4**-GABA C-2 (triplet), **5**- glutamate C-4 (triplet) **6**-succinate C-2/3 (singlet), **7**- aspartate C-3 (doublet of doublet), **8**- creatine C-4 (singlet), **9**- taurine C-2 (triplet) **10**- creatine phosphate (singlet), **11**-myoinositol C-1 (triplet), **12**- lactate C-2 (quadraplet) **13**- N-acetyl aspartate C-6 (singlet), **14**- β -glucose **15** - α -glucose, **16**- fumarate C-2/3 (singlet) **17**- Formate C-1 (singlet) **18**-ADP&ATP (imidazolic H) (doublet) **19**- AMP (imidazolic H) (singlet) **20** -NADH (H on 2 position on the nicotinic ring) (singlet) **21**- NAD $^+$ (H on 2 position on the nicotinic ring) (singlet).

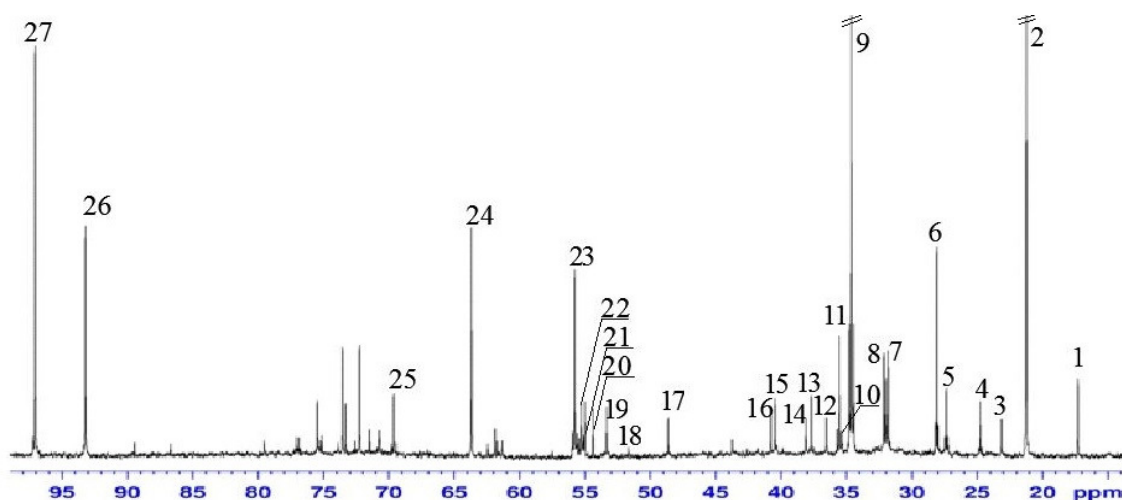


Figure 6: Typical ^{13}C -NMR spectrum of methanol-chloroform extract from the limbic structures of rats. The animals were administered an intraperitoneal injection of $[1-^{13}\text{C}]$ glucose and $[1,2-^{13}\text{C}]$ acetate, and were microwave irradiated 15 mins later. The singlets denote monolabeled metabolites mainly derived from $[1-^{13}\text{C}]$ glucose whereas doublets denote double-labeled metabolites mainly originating from the metabolism of $[1,2-^{13}\text{C}]$ acetate. The following peaks were identified and quantified: 1-alanine C-3, 2-lactate C-3, 3-N-acetyl aspartate C-6, 4-GABA C-3, 5-glutamine C-3, 6-glutamate C-3, 7-glutamine C-4, 8-glutamate C-4, 9-glutamate C-4, 10-succinate C-2/3 11-GABA C-2, 12-aurine C-2, 13-aspartate C-3, 14—creatine C-2, 15—GABA C-4, 16-N-acetyl aspartate C-3, 17-aurine C-1, 18-Alanine C-2, 19— aspartate C-2, 20-N-acetyl aspartate C-2, 21-creatine C-4, 22-glutamine C-2, 23-glutamate C-2, 24— ethylene glycol, 25-lactate C-2, 26- α -glucose, 27- β -glucose.

Labeling pattern of $[1-^{13}\text{C}]$ glucose

$[1-^{13}\text{C}]$ glucose is converted into one molecule of $[3-^{13}\text{C}]$ pyruvate and one molecule of unlabelled pyruvate via glycolysis. $[3-^{13}\text{C}]$ pyruvate is then either converted to $[3-^{13}\text{C}]$ lactate or $[3-^{13}\text{C}]$ alanine in the cytosol or converted in to $[2-^{13}\text{C}]$ acetyl CoA by PDH in the mitochondria of both neurons and astrocytes. $[2-^{13}\text{C}]$ acetyl CoA condenses with oxaloacetate and enters the TCA cycle. After several enzymatic reactions, $[4-^{13}\text{C}]$ α -ketoglutarate is formed and can be converted into $[4-^{13}\text{C}]$ glutamate, $[4-^{13}\text{C}]$ glutamine, and $[2-^{13}\text{C}]$ GABA. If $[4-^{13}\text{C}]$ α -ketoglutarate continues in the cycle, the ^{13}C label will be divided into equal parts of $[2-^{13}\text{C}]$ or $[3-^{13}\text{C}]$ succinate and then $[2-^{13}\text{C}]$ or $[3-^{13}\text{C}]$ oxaloacetate which finally can give rise to $[2-^{13}\text{C}]$ or $[3-^{13}\text{C}]$ aspartate or $[2-^{13}\text{C}]$ or $[3-^{13}\text{C}]$ glutamate/glutamine and $[3-^{13}\text{C}]$ or $[4-^{13}\text{C}]$ GABA by condensing with unlabelled acetyl CoA in the 2nd turn of the TCA cycle. In astrocytes, $[3-^{13}\text{C}]$ pyruvate can also be carboxylated by PC to $[3-^{13}\text{C}]$ oxaloacetate which can result in the formation of $[2-^{13}\text{C}]$ glutamate, $[2-^{13}\text{C}]$ glutamine and $[4-^{13}\text{C}]$ GABA (Sonnewald & Kondziella 2003, Zwingmann & Leibfritz 2003, Melo *et al.* 2007, Morken *et al.* 2013). The labeling pattern for the first turn of the TCA cycle is shown in Figure 7 (Melo *et al.* 2007).

Labeling pattern of [1,2-¹³C]acetate

[1,2-¹³C]acetate is taken up by astrocytes and converted into [1,2-¹³C]acetyl CoA which then enters into the TCA cycle and gives rise to [4,5]-¹³C glutamate, [4,5-¹³C] glutamine and [1,2-¹³C]GABA. In the 2nd turn of the TCA cycle, labeling will lead to glutamate and glutamine equally labelled in either the [1,2-¹³C] or [3-¹³C] positions of glutamate/glutamine and [3-¹³C] or [4-¹³C] positions of GABA (Zwingmann & Leibfritz 2003, Melo *et al.* 2007, Morcken *et al.* 2013).

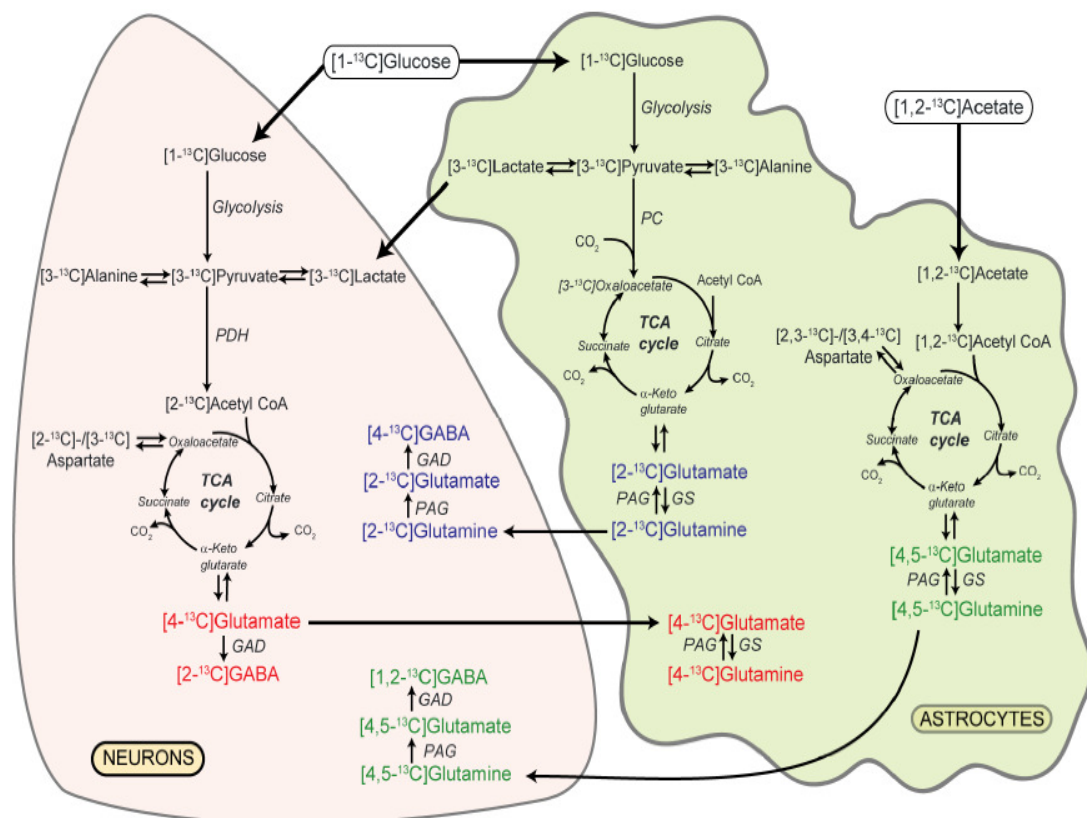


Figure 7: Labeling patterns derived from injection of [1-¹³C]glucose and [1,2-¹³C]acetate into rats in neurons and astrocytes after glycolysis and in the first turn of the TCA cycle. For simplicity, the enzyme PDH is not shown in the astrocytes. In addition, metabolites from the 2nd turn of the TCA cycle are not shown. The figure is taken from (Melo *et al.* 2007). Abbreviations: GAD, glutamic acid decarboxylase; GS, glutamine synthetase; PAG, phosphate-activated glutaminase; PC, pyruvate carboxylase; TCA: tricarboxylic acid.

3.1.7.2. Metabolic ratios

% ¹³C Enrichment

Percent ¹³C enrichment is calculated from the amounts of label corrected for natural abundance over the total amount of metabolite

Acetate/Glucose utilisation ratio

It is a ratio that estimates the relative contribution of acetate and glucose to the labelling of glutamate, glutamine and GABA formation. The acetate vs glucose utilization ratio is expressed as:

$$= \frac{[4,5-^{13}\text{C}]\text{glutamate (glutamine)}}{[4-^{13}\text{C}]\text{glutamate (glutamine)}}$$

and

$$= \frac{[1,2-^{13}\text{C}]\text{GABA}}{[2-^{13}\text{C}]\text{GABA}}$$

[1,2-¹³C]acetate incorporation results in [4,5-¹³C] glutamate, [4,5-¹³C]glutamine, and [1,2-¹³C]GABA, whereas [1-¹³C]glucose yields [4-¹³C]glutamate, [4-¹³C]glutamine, and [2-¹³C]GABA (Morken *et al.* 2013).

The ¹³C cycling ratio

The ¹³C cycling ratio gives an indication of how long the label stays in the TCA cycle before it is incorporated into glutamate, glutamine and other metabolites. The cycling ratio for ¹³C from [1,2-¹³C]acetate is expressed as:

$$= \frac{2 * [1,2-^{13}\text{C}]\text{glutamate (glutamine)}}{[4,5-^{13}\text{C}]\text{glutamate (glutamine)}}$$

The cycling ratio for ¹³C from [1-¹³C]glucose is calculated as:

$$= \frac{2 * ([3-^{13}\text{C}]\text{glutamate (glutamine)}) - [1,2-^{13}\text{C}]\text{glutamate (glutamine)}}{[4-^{13}\text{C}]\text{glutamate (glutamine)}}$$

(Sonnewald & Kondziella 2003).

Pyruvate carboxylase activity

PC is a mitochondrial enzyme that is found only in astrocytes. It converts pyruvate into oxaloacetate (reviewed in (Sonnewald & Rae 2010)). PC activity in astrocytes leads to labelling in the [2-¹³C] positions of glutamate and glutamine and in the [4-¹³C] positions of GABA, however this process is not unique for PC and in the second turn of TCA cycle equal amounts of ¹³C labelling occurs at [2-¹³C] and [3-¹³C] positions of glutamate and glutamine and at [3-¹³C] and [4-¹³C] positions of GABA from [1-¹³C]glucose. Therefore when PC activity is calculated, ¹³C labelling from [3-¹³C] positions of glutamate, glutamine and GABA should be subtracted from [2-¹³C] positions of glutamate (glutamine) and [4-¹³C] positions of GABA (Morken *et al.* 2013). However, following PC activity, oxaloacetate may be converted into fumarate. This pathway is called oxaloacetate-fumarate-flux or backflux. Therefore the results obtained for PC activity are an underestimation if backflux is not considered. For precise calculation of PC label, the amount of PC label through backflux has to be considered (Brekke *et al.* 2012).

The amounts of metabolites through PC activity can be estimated using the following equation:

$$= [2-^{13}\text{C}]\text{glutamate (glutamine)} - ([3-^{13}\text{C}]\text{glutamate (glutamine)} - [1,2-^{13}\text{C}]\text{glutamate (glutamine)})$$

Pyruvate carboxylase/Pyruvate dehydrogenase (PC/PDH) ratio

The ratio of flux through PC (present only in astrocytes) versus the flux through PDH (Present in both neurons and astrocytes) gives information about the replacement of intermediates lost from the TCA cycle.

The PC/PDH ratio for glutamate and glutamine from [1-¹³C]glucose metabolism is calculated as:

$$= \frac{[2-^{13}\text{C}]\text{glutamate (glutamine)} - ([3-^{13}\text{C}]\text{glutamate (glutamine)} - [1,2-^{13}\text{C}]\text{glutamate (glutamine)})}{[4-^{13}\text{C}]\text{glutamate (glutamine)}}$$

The labelling of GABA from the second turn is identical for [1-¹³C]glucose and [1,2-¹³C]acetate (Morken *et al.* 2013).

Transfer ratio from astrocytes to neurons

Since most of the glutamate in the brain is located in neurons (Ottersen & Storm-Mathisen, 1985) and acetate is metabolized in astrocytes (Sonnewald *et al.* 1993), significant amount of labelling of [4,5-¹³C]glutamate from [1,2-¹³C]acetate can only happen with the transfer of [4,5-¹³C]glutamine from the astrocytic to the neuronal compartment. Therefore, a ratio between [4,5-¹³C]glutamate and [4,5-¹³C]glutamine would show the transfer from astrocytes to neurons. The transfer of substrate from astrocytes to glutamatergic neurons is calculated as:

$$= \frac{\text{Amount of [4,5-}^{13}\text{C]glutamate}}{\% \text{ enrichment of [4,5-}^{13}\text{C]glutamine}}$$

Similarly, an estimate of the transfer of substrate from astrocytes to GABAergic neurons can be made as:

$$= \frac{\text{Amount of [1,2-}^{13}\text{C]GABA}}{\% \text{ enrichment of [4,5-}^{13}\text{C]glutamine}}$$

(Morken *et al.* 2013).

3.2.8. Data and statistical analysis

TopSpin 3.0 software (Bruker BioSpin GmbH) was used to identify and integrate relevant peaks in the ¹³C and ¹H NMR spectra. Quantification of metabolites was done from the integrals of the peak areas using TSP and ethylene glycol as internal standards for the ¹H and ¹³C spectra, respectively. The total amounts (¹³C plus ¹²C) of glutamate, glutamine, GABA, aspartate, glutathione and taurine in the extracts were quantified using HPLC. Amounts from the ¹H spectra were corrected for the number of protons constituting the peak and for ¹³C containing metabolites. ¹³C-labeled metabolites were corrected for nuclear Overhauser and relaxation effects. In addition, singlets in the ¹³C spectra were corrected for 1.1% natural abundance of ¹³C. When the metabolite was double-labelled, ¹³C natural abundance was calculated as 1.1%×1.1% of total metabolite amount. All amounts were corrected for tissue weight and also 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. All results are expressed as mean ± SEM. Statistical differences between the groups were analyzed using ANOVA followed by LSD multiple comparison test. The level of significance was set at p < 0.05.

4. Results

4.1. Glycolysis related metabolites - [1-¹³C]glucose metabolism

The total concentrations of metabolites related to glycolysis – glucose, lactate and alanine were quantified using ¹H-NMR spectroscopy (Table 1). Through glycolysis, [1-¹³C]glucose is converted to [3-¹³C]pyruvate, which can either be converted to [3-¹³C]lactate, [3-¹³C]alanine or [2-¹³C]acetyl CoA (Fig. 7). Although there is a trend towards an increase, total and labeled amounts of glucose, lactate and alanine in the UT-TLE rats compared to the control rats, statistical significance was not reached.

Table 1. Total amounts of glucose, lactate and alanine (nmol/g), % ¹³C enrichment and amounts with [1-¹³C]glucose, [3-¹³C]lactate, and [3-¹³C]alanine in extracts from limbic structures of the control, UT- TLE, CRS- TLE and CRS-ALE rats.

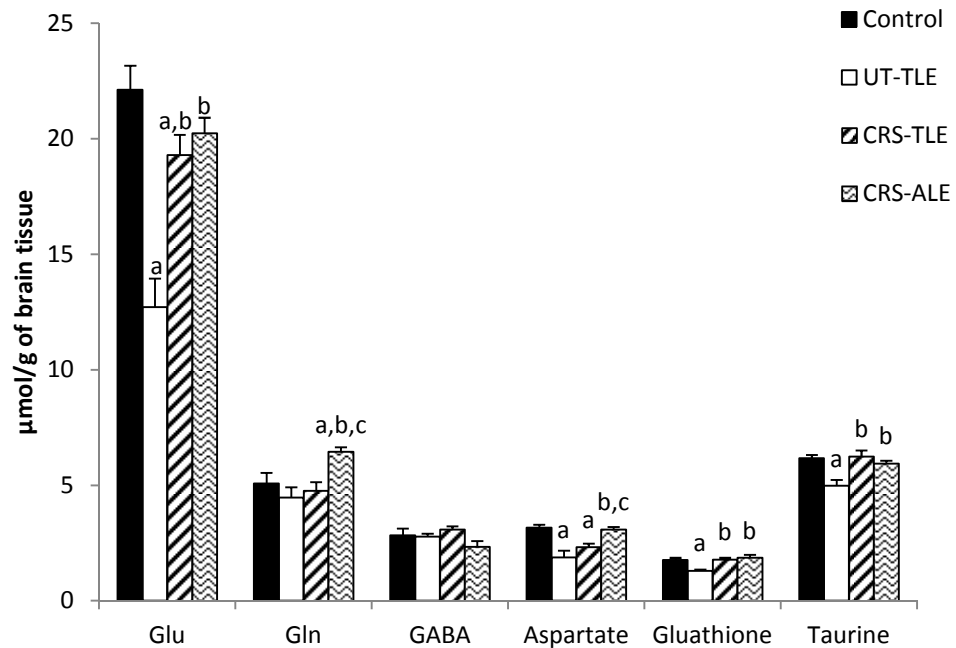
	Control n=6	UT-TLE n=4	CRS-TLE n=9	CRS-ALE n=6
Total Glucose	3.8±0.8	6.2±0.7	4.9±0.6	4.7±0.3
[1-¹³C]glucose	1.2±0.3	2.0±0.3	1.5±0.2	1.6±0.2
% ¹³C enrichment	29.4±3.9	32.6±2.3	31.4±2.0	35.4±1.9
Total Lactate	10.4±3.6	11.7±4.5	9.4±3.3	3.3±0.2
[3-¹³C]lactate	1.2±0.4	1.2±0.5	1.0±0.4	0.3±0.0
% ¹³C enrichment	10.9±1.0	9.6±1.6	8.1±1.1	7.5±1.1
Tot Alanine	1.3±0.2	1.4±0.2	1.0±0.1	1.0±0.1
[3-¹³C]alanine	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
% ¹³C enrichment	8.9±1.3	6.5±1.7	7.8±0.9	7.5±1.4

Rats were injected with [1-¹³C]glucose and [1,2-¹³C]acetate intraperitoneally and brains were microwave irradiated 15 minutes later (for details see Materials and Methods). Results were obtained using ¹H NMR and ¹³C NMR spectroscopy analyses of brain extracts of limbic structures from the control rats, untreated temporal lobe epileptic rats (UT-TLE), carisbamate treated temporal lobe epileptic (CRS-TLE) and absence like epileptic (CRS-ALE) rats two months after injection of lithium-pilocarpine. The control group received vehicle. ¹³C isotopomers were corrected for natural abundance. Values are presented as mean ± SEM. Statistical analyses were performed using ANOVA followed by LSD test. Abbreviations: UT-TLE: untreated temporal lobe epileptic rats; CRS-TLE: carisbamate treated temporal lobe epileptic rats; CRS-ALE: carisbamate treated absence like epileptic rats.

4.2. TCA cycle related metabolites and other amino acids measured by HPLC

The total concentrations of metabolites (glutamate, glutamine, GABA, aspartate, glutathione and taurine) in the limbic structures were measured using HPLC.

Figure 8. The concentrations ($\mu\text{mol/g}$ of brain tissue) of glutamate, glutamine, GABA, aspartate, glutathione and taurine in limbic structures obtained using HPLC.



Total amounts of metabolites in brain extracts of limbic areas ($\mu\text{mol/g}$ of brain tissue) after intraperitoneal administration of saline, lithium-pilocarpine, and carisbamate (90mg/Kg). The total amounts of metabolites were determined with HPLC (for details see Materials and Methods). Data represent mean \pm SEM, and the superscripts (a,b and c) indicate statistically significant difference to the control group, the UT-TLE group and the CRS-TLE group respectively ($p < 0.05$). Statistical analyses were performed using ANOVA followed by LSD test. $n=7$ (Control), $n=4$ (UT-TLE), $n=11$ (CRS-TLE), $n=7$ (CRS-ALE) Abbreviations: UT-TLE: untreated temporal lobe epileptic rats; CRS-TLE: carisbamate treated temporal lobe epileptic rats CRS-ALE: carisbamate treated absence like epileptic rats Glu: glutamate Gln: glutamine GABA: gamma amino butyric acid.

There was a decrease in the amounts of glutamate, aspartate, glutathione and taurine in the UT-TLE rats compared to the control rats. CRS treated rats (both TLE and ALE) showed an increase in the amounts of glutamate, glutathione and taurine compared to the UT-TLE group. Glutamine content was actually increased significantly in the CRS-ALE rats compared to the control, UT-TLE, and CRS-TLE groups. CRS-ALE rats displayed increased levels of glutamine and aspartate compared to the CRS-TLE rats. No significant difference was observed in the total amount of GABA among all groups.

4.3. Other Metabolites from $^1\text{H-NMR}$ spectroscopy

Total amount of energy related metabolites in brain extracts of limbic structures was measured using ^1H NMR spectroscopy (see Table 2).

Table 2. Total amounts of energy related metabolites in brain extracts of limbic structures ($\mu\text{mol/g}$ of brain tissue) obtained using ^1H NMR spectroscopy.

	Control n=6	UT-TLE n=4	CRS-TLE n=9	CRS-ALE n=6
NAD+	0.16 \pm 0.06	0.09 \pm 0.04	0.28 \pm 0.06	0.28 \pm 0.06
NADH	0.30 \pm 0.08	0.31 \pm 0.06	0.18 \pm 0.06	0.17 \pm 0.06
AMP	0.36 \pm 0.08	0.31 \pm 0.02	0.38 \pm 0.09	0.43 \pm 0.07
ATP	0.49 \pm 0.20	0.34 \pm 0.09	0.72 \pm 0.13	0.68 \pm 0.14
ADP	0.57 \pm 0.23	0.37 \pm 0.08	1.16 \pm 0.26	1.07 \pm 0.31
Formate	0.06 \pm 0.00	0.06 \pm 0.00	0.07 \pm 0.01	0.07 \pm 0.01
Fumarate	0.07 \pm 0.02	0.06 \pm 0.02	0.04 \pm 0.01	0.03 \pm 0.01
Myoinositol	10.94 \pm 0.60	11.16 \pm 0.58	11.65 \pm 0.38	11.26 \pm 0.17
Creatine Pho.	0.90 \pm 0.38	0.66 \pm 0.38	1.88 \pm 0.43	2.13 \pm 0.30
Creatine	6.50 \pm 0.72	5.69 \pm 0.93	5.42 \pm 0.46	5.23 \pm 0.29
Succinate	0.06 \pm 0.01	0.04 \pm 0.02	0.05 \pm 0.01	0.06 \pm 0.01
NAA	9.01 \pm 0.27	5.33 \pm 0.26 ^a	6.67 \pm 0.32 ^{a,b}	8.68 \pm 0.32 ^{b,c}

Results were obtained using ^1H NMR spectroscopy analyses of brain extracts of limbic structures from the control, UT-TLE, CRS-TLE and CRS-ALE rats two months after injection of lithium-pilocarpine. The control groups received vehicle. Data are presented as mean \pm SEM, and the superscripts (a,b and c) indicate a statistically significant difference to the control group, the UT-TLE group and the CRS-TLE group respectively ($p < 0.05$). Statistical analyses were performed using ANOVA followed by LSD test. Abbreviations: UT-TLE: untreated temporal lobe epileptic rats; CRS-TLE: carisbamate treated temporal lobe epileptic rats; CRS-ALE: carisbamate treated absence like epileptic rats; NAD+/NADH: nicotinamide adenine dinucleotide; AMP: adenosine monophosphate; ADP: adenosine diphosphate; ATP: adenosine triphosphate; Creatine Pho: creatine phosphate; NAA: N-acetylaspartate.

We measured the amounts of NAD+, NADH, AMP, ATP, ADP, formate, fumarate, myoinositol, creatine phosphate, creatine, succinate and N-acetylaspartate (NAA) from ^1H spectra in limbic structures (ethorinal/piriform cortex and amygdala). The only difference found was in the levels of NAA. There was a decrease in the concentration of NAA in the UT-TLE and CRS-TLE rats compared to the control group. However, treatment with CRS (in both the CRS-TLE and CRS-ALE groups) prevented the decrease in the concentration of NAA compared to the UT-TLE rats. CRS-ALE rats showed a similar level of NAA as control rats and had a higher level of NAA compared with the CRS-TLE rats.

4.4. ¹³C Labeled TCA cycle related metabolites derived from [1-¹³C]glucose and [1,2-¹³C]acetate

Labeling of relevant metabolites (glutamate, glutamine and GABA) derived from [1-¹³C]glucose and [1,2-¹³C]acetate are presented in Table 3.

Table 3. Amounts (nmol/g of brain tissue) of ¹³C labeled isotopomers of glutamate, glutamine, and GABA in the limbic structures derived from [1-¹³C]glucose and [1,2-¹³C]acetate.

Metabolite	Control n=6	UT-TLE n=4	CRS-TLE n=9	CRS-ALE n=6
[4- ¹³ C]glutamate	739.3±26.7	366.1±64.6 ^a	609.8±38.4 ^{a,b}	601.2±45.9 ^{a,b}
[4- ¹³ C]glutamine	121.7±14.3	60.5±8.9 ^a	122.2±10.9 ^b	111.9±6.1 ^b
[2- ¹³ C]GABA	112.8±19.6	67.4±10.6	90.6±11.9	71.1±7.7
[2- ¹³ C]glutamate	126.3±6.2	55.5±14.9 ^a	92.0±15.1	72.3±13.7 ^a
[2- ¹³ C]glutamine	63.2±10.3	36.7±6.4	67.8±6.7	60.0±3.1
[3- ¹³ C]glutamate	80.9±6.0	42.6±15.8	64.4±8.7	38.1±15.0
[3- ¹³ C]glutamine	55.8±7.3	38.4±9.1	60.8±4.3	56.5±3.4
[3- ¹³ C]GABA	22.9±6.2	7.0±4.2	16.2±3.6	12.7±1.6
[4- ¹³ C]GABA	36.7±9.0	14.9±4.9	29.8±4.5	19.7±1.2
[4,5- ¹³ C]glutamate	331.8±51.1	203.0±37.7 ^a	202.8±23.8 ^a	194.0±16.5 ^a
[4,5- ¹³ C]glutamine	377.3±42.0	265.2±54.0	278.9±21.6	437.5±43.0 ^{b,c}
[1,2- ¹³ C]GABA	51.8±10.4	32.2±5.7	27.5±4.8 ^a	23.1±3.3 ^a
[1,2- ¹³ C]glutamate	65.7±9.7	43.6±7.6	28.6±5.3 ^a	46.1±4.4
[1,2- ¹³ C]glutamine	72.5±7.8	54.0±2.9	63.3±9.0	70.6±6.2

Rats were injected with [1-¹³C]glucose and [1,2-¹³C]acetate intraperitoneally and microwave fixed 15 minutes later (for details see Materials and Methods). Results were obtained using ¹³NMR spectroscopy analyses of brain extracts of limbic structures from the control, UT-TLE, CRS-TLE and CRS-ALE rats two months after injection of lithium-pilocarpine. The control group received vehicle. Data are presented as mean ± SEM, and the superscripts (a,b and c) indicate a statistically significant difference to the control group, the UT-TLE group and the CRS-TLE group respectively (p < 0.05). Statistical analyses were performed using ANOVA followed by LSD test. Abbreviations: UT-TLE: untreated temporal lobe epileptic rats; CRS-TLE: carisbamate treated temporal lobe epileptic rats; CRS-ALE: carisbamate treated absence like epileptic rats. GABA: gamma amino butyric acid.

Metabolites formed during the first turn of the TCA cycle after [1-¹³C]glucose injection include [4-¹³C]glutamate, [4-¹³C]glutamine and [2-¹³C]GABA (see Fig. 7). The amounts of [4-¹³C]glutamate were reduced in the UT-TLE, CRS-TLE and CRS-TLE rats compared to the control rats. Treated CRS-TLE and CRS-ALE rats showed an increase in [4-¹³C]glutamate compared to the UT-TLE rats. The levels of [4-¹³C]glutamine were reduced in the UT-TLE rats compared to the control rats.

However, CRS treatment kept [4-¹³C]glutamine levels from dropping and treated rats showed a [4-¹³C]glutamine increase compared to the UT-TLE rats. No statistically significant difference was observed in the levels of [2-¹³C] GABA.

Equal amounts of [2-¹³C]glutamate/glutamine and [3-¹³C]glutamate/glutamine and equal amounts of [3-¹³C]GABA and [4-¹³C]GABA can be formed in the 2nd turn of the TCA cycle. The level of [2-¹³C]glutamate was reduced in the UT-TLE rats compared to the control rats. CRS-ALE rats showed increased [2-¹³C]glutamate compared to the untreated ones, without a concomitant change in the corresponding isotopomers with ¹³C label in the C-3 position. No significant difference was observed in other metabolites which can be obtained from the 2nd turn of the TCA cycle. However, [2-¹³C]glutamate, [2-¹³C]glutamine, and [4-¹³C]GABA can also be obtained through pyruvate carboxylation in astrocytes. The amount of glutamate from PC (see Materials and Methods) activity was reduced in the UT-TLE rats compared to the control rats (Table 4). CRS-ALE rats showed similar PC activity with controls. PC/PDH ratio in glutamate however showed no significant difference (see Table 6). No significant difference was observed in the amounts of labeling of glutamine and GABA through PC.

Table 4. Amounts of pyruvate carboxylase (PC) activity.

		Control	UT-TLE	CRS-TLE	CRS-ALE
	Metabolite	n=6	n=4	n=9	n=6
PC	Glutamate	111.1±14.4	56.5±7.4 ^a	59.8±9.9 ^a	80.3±8.4
	Glutamine	79.9±12.3	52.3±3.9	70.3±14.1	74.1±7.9
	GABA	13.8±4.2	7.9±3.7	13.6±3.3	6.9±1.8

Rats were injected with [1-¹³C]glucose and [1,2-¹³C]acetate intraperitoneally and microwave irradiated 15 minutes later (for details see Materials and Methods). Results were obtained using ¹³NMR spectroscopy analyses of brain extracts of limbic structures from the control, UT-TLE, CRS-TLE and CRS-ALE rats two months after injection of lithium-pilocarpine. The control group received vehicle. Data are presented as mean ± SEM, and the superscripts (a,b and c) indicate a statistically significant difference to the control group, the UT-TLE group and the CRS-TLE group respectively (p < 0.05). Statistical analyses were performed using ANOVA followed by LSD test. Abbreviations: UT-TLE: untreated temporal lobe epileptic rats; CRS-TLE: carisbamate treated temporal lobe epileptic rats; CRS-ALE: carisbamate treated absence like epileptic rats; GABA: gamma amino butyric acid; PC: pyruvate carboxylase.

Since acetate is preferentially taken up by astrocytes (Waniewski & Martin 1998), labelling from [1,2-¹³C]acetate shows astrocytic metabolism. [1,2-¹³C]acetate is taken up by astrocytes and converted into [1,2-¹³C]acetyl CoA which then enters into the TCA cycle and gives rise to [4,5-¹³C]glutamate and [4,5-¹³C]glutamine (see Fig.7). [4,5-¹³C]glutamine can be transported into neurons and then converted into [4,5-¹³C]glutamate by PAG. [4,5-¹³C]glutamate can then be converted into [1,2-¹³C]GABA by GAD in GABAergic neurons. The levels of [4,5-¹³C]glutamate was reduced in all the UT-TLE, CRS-TLE and CRS-ALE groups compared to the control group. Treatment with CRS didn't change the level of [4,5-¹³C]glutamate, however it was able to increase [4,5-¹³C]glutamine levels in the CRS-ALE group compared to the UT-TLE and CRS-TLE groups. Although it was not statistically significant, the amount of [4,5-¹³C]glutamine appears to be reduced in the UT-TLE (p=0.07) and CRS-TLE (p=0.05) rats. In addition, treatment with CRS reduced the level of [1,2-¹³C]GABA in the CRS-TLE and CRS-ALE rats compared to the control rats. From the amount, it appears that there is a reduction in [1,2-¹³C]GABA in the UT-TLE rats but it was not statistically significant (p=0.07). There were no differences among groups in metabolites obtained from the 2nd turn of TCA cycle from [1,2-¹³C]acetate except in [1,2-¹³C]glutamate which was reduced in the CRS-TLE rats.

4.5. % ¹³C enrichment of metabolites

Table 5. % ¹³C enrichment with metabolites derived from [1-¹³C]glucose and [1,2-¹³C]acetate.

Metabolite	Control n=6	UT-TLE n=4	CRS-TLE n=9	CRS-ALE n=6
[4- ¹³ C]glutamate	3.3±0.1	2.9±0.5	3.3±0.3	3.0±0.3
[4- ¹³ C]glutamine	2.4±0.1	1.4±0.3 ^a	2.5±0.2 ^b	1.7±0.1 ^{a,c}
[2- ¹³ C]GABA	3.7±0.5	2.4±0.4	3.0±0.3	3.1±0.3
[2- ¹³ C]glutamate	0.6±0.0	0.4±0.1	0.5±0.1	0.4±0.1
[2- ¹³ C]glutamine	1.2±0.1	0.9±0.2	1.3±0.1	0.9±0.1
[3- ¹³ C]glutamate	0.4±0.0	0.3±0.1	0.4±0.1	0.2±0.1
[3- ¹³ C]glutamine	1.1±0.1	0.9±0.2	1.2±0.1	0.9±0.0
[3- ¹³ C]GABA	0.8±0.2	0.2±0.1	0.5±0.1	0.6±0.1
[4- ¹³ C]GABA	1.2±0.3	0.5±0.2	1.0±0.1	0.9±0.1
[4,5- ¹³ C]glutamate	1.5±0.2	1.6±0.2	1.1±0.1 ^b	1.0±0.1 ^{a,b}
[4,5- ¹³ C]glutamine	7.6±0.6	6.1±1.2	5.6±0.3	6.8±0.8
[1,2- ¹³ C]GABA	1.7±0.3	1.2±0.2	0.9±0.1 ^a	1.0±0.1 ^a
[1,2- ¹³ C]glutamate	0.3±0.0	0.3±0.0	0.2±0.0 ^{a,b}	0.2±0.0 ^b
[1,2- ¹³ C]glutamine	1.5±0.1	1.2±0.1	1.3±0.2	1.1±0.1

Rats were injected with [1-¹³C]glucose and [1,2-¹³C]acetate intraperitoneally and microwave irradiated 15 minutes later (for details see Materials and Methods). Results were obtained and analyzed using ¹³NMR spectroscopy of analyses of brain extracts of limbic structures from the control, UT-TLE, CRS-TLE and CRS-ALE rats two months after injection of lithium-pilocarpine. The control groups received vehicle. Data are presented as mean ± SEM, and the superscripts (a,b and c) indicate a statistically significant difference to the control group, the UT-TLE group and the CRS-TLE group respectively (p < 0.05). Statistical analyses were performed using ANOVA followed by LSD test. Abbreviations: UT-TLE: untreated temporal lobe epileptic rats; CRS-TLE: carisbamate treated temporal lobe epileptic rats; CRS-ALE: carisbamate treated absence like epileptic rats; GABA: gamma amino butyric acid.

Percent ¹³C enrichment with different isotopomers of glutamate, glutamine and GABA is calculated from the amounts of the label corrected for natural abundance over the total amount of the metabolite. There was a decrease in the % ¹³C enrichment with [4-¹³C]glutamine in the UT-TLE and CRS-ALE rats compared to the control rats. % ¹³C enrichment with [4-¹³C]glutamine in the CRS-TLE group was higher than the UT-TLE and CRS-ALE groups. The % ¹³C enrichment with [4-¹³C]glutamate and [2-¹³C]GABA was unchanged.

The % ¹³C enrichment with [4,5-¹³C]glutamate was reduced in the CRS-TLE and CRS-ALE groups compared to the UT-TLE rats. CRS-ALE rats had also reduced % ¹³C enrichment with the [4,5-¹³C]glutamate levels compared to the control group. %

^{13}C enrichment with $[1,2-^{13}\text{C}]\text{GABA}$ was reduced in both CRS-TLE and CRS-ALE rats compared to control rats. The % ^{13}C enrichment with $[1,2-^{13}\text{C}]\text{glutamate}$ which is derived from the 2nd turn of the TCA cycle in astrocytes was lower in the CRS-TLE and CRS-ALE rats compared to the UT-TLE rats. CRS-TLE rats had also reduced % ^{13}C enrichment with $[1,2-^{13}\text{C}]\text{glutamate}$ compared to the control rats.

4.6. Metabolic ratios

Table 6. TCA cycling, acetate vs glucose utilization, PC/PDH, astrocytes to neurons transfer, and neurons to astrocytes transfer.

Ratios	Metabolites	Control n=6	UT-TLE n=4	CRS-TLE n=9	CRS-ALE n=6
Glucose 2nd/1st	Glutamate	0.12±0.02	0.12±0.48	0.17±0.02	0.05±0.08 ^c
	Glutamine	0.32±0.06	0.4±0.16	0.46±0.09	0.40±0.06
	GABA	0.4±0.06	0.15±0.09	0.33±0.05	0.35±0.04
Acetate 2nd/1st	Glutamate	0.4±0.04	0.43±0.05	0.29±0.06	0.5±0.03 ^c
	Glutamine	0.38±0.03	0.45±0.06	0.46±0.06	0.3±0.0
	GABA	0.85±0.13	0.38±0.22	1.32±0.26	1.27±0.28
PC/PDH	Glutamate	0.17±0.02	0.15±0.03	0.1±0.02	0.12±0.02
	Glutamine	0.65±0.07	0.93±0.2	0.66±0.18	0.7±0.09
	GABA	0.13±0.03	0.1±0.04	0.17±0.03	0.12±0.04
Acetate/ Glucose	Glutamate	0.43±0.06	0.58±0.03	0.32±0.04 ^b	0.35±0.02 ^b
	Glutamine	3.12±0.19	4.4±0.56 ^a	2.43±0.25 ^b	3.96±0.35 ^c
	GABA	0.47±0.05	0.48±0.05	0.32±0.07	0.35±0.04
Astrocyte to Neuron	4,5glu/%4,5gln	44.0±7.2	35.1±5.0	36.6±3.7	28.9±1.0
	1,2GABA/%4,5gln	6.7±1.2	5.4±0.4	4.9±0.8	3.4±0.4
Neuron to Astrocyte	4gln/%4glu	36.3±3.4	21.2±1.2 ^a	36.7±1.7 ^b	38.6±2.8 ^b

Rats were injected with $[1-^{13}\text{C}]\text{glucose}$ and $[1,2-^{13}\text{C}]\text{acetate}$ intraperitoneally and microwave fixed 15 minutes later (for details see Materials and Methods). Results were obtained and analyzed using ^{13}NMR spectroscopy of analyses of brain extracts of limbic structures from the control, UT-TLE, CRS-TLE and CRS-ALE rats two months after injection of lithium-pilocarpine. The control groups received vehicle. For calculations of the ratios see Materials and Methods. Data are presented as mean ± SEM, and the superscripts (a,b and c) indicate a statistically significant difference to the control group, the UT-TLE group and the CRS-TLE group respectively ($p < 0.05$). Statistical analyses were performed using ANOVA followed by LSD test. Abbreviations: UT-TLE: untreated temporal lobe epileptic rats; CRS-TLE: carisbamate treated temporal lobe epileptic rats; CRS-ALE: carisbamate treated absence like epileptic rats; GABA: gamma aminobutyric acid; Gln: glutamine; Glu: glutamate

The ^{13}C cycling ratio (see Materials and Methods) gives an indication of how long the label stays in the TCA cycle before it is incorporated into glutamate, glutamine and other metabolites. TCA cycling ratio in glutamate with $[1-^{13}\text{C}]\text{glucose}$ as a precursor was reduced in the CRS-ALE rats compared to the CRS-TLE rats which shows more

of the ^{13}C label from $[1-^{13}\text{C}]$ glucose found in glutamate was derived from the 1st turn. While, increased TCA cycling ratio in glutamate in the CRS-ALE rats with $[1,2-^{13}\text{C}]$ acetate as a precursor compared to the CRS-TLE rats was observed which shows more of the ^{13}C label from $[1,2-^{13}\text{C}]$ acetate is found from the 1st turn.

The acetate/glucose utilization ratio (see Materials and Methods) estimates the relative contribution of acetate and glucose to the labelling of glutamate, glutamine and GABA. The acetate/glucose utilization ratio for glutamate was lowered for the CRS-TLE and CRS-ALE groups compared to the UT-TLE group. However, the ratio for glutamine was increased in the UT-TLE rats compared to the control rats and decreased for the CRS-TLE rats compared to the UT-TLE rats. CRS-ALE groups had increased acetate/glucose utilization ratio compared to the CRS-TLE group.

The transfer of glutamate from glutamatergic neurons to astrocytes was increased in CRS treated groups compared to the UT-TLE rats. However, the transfer of glutamine from astrocytes to glutamatergic and GABAergic neurons was unchanged.

5. Discussion

5.1. Glycolysis related metabolites

Glucose metabolism

The brain depends on glucose and oxygen to meet its energy demand and for the synthesis of metabolites such as glutamate, glutamine and GABA. Glucose is converted into pyruvate through glycolysis. Pyruvate then can be converted into acetyl CoA (by PDH), lactate (by LDH) and alanine (by alanine aminotransferase) (McKenna *et al.* 2012).

Glucose hypometabolism is one typical feature that is observed in chronic models of TLE including the lithium-pilocarpine and kainic acid models (Jupp *et al.* 2012, Lee *et al.* 2012). It was also observed to precede limbic atrophy in rat model of TLE which was independent of neuronal loss (Jupp *et al.* 2012). Glucose metabolism in the entire limbic area was reduced during the latent period of epileptogenesis in the lithium-pilocarpine model (Lee *et al.* 2012). In addition, interictal hypometabolism in limbic areas was observed during the chronic phase of TLE (Dube *et al.* 2001). Increased labeled glucose content in epileptic rats normally shows decreased glucose utilization (Alvestad *et al.* 2011). However, in our study no significant difference was observed in glucose metabolism although there was a trend towards increased level of glucose (38.7%), lactate and alanine in the UT-TLE rats compared to the control rats and increased values by CRS treatment following lithium-pilocarpine (see Table 1). Glycolysis could have been reduced in the epileptic rats, as reduced labeling in glutamate and glutamine (see Table 3) is suggestive of decreased formation of acetyl CoA from [1-¹³C]glucose. The reason for not finding statistically significant differences might be ascribed to the large SEM as there were few animals in the UT-TLE group since two died. Similar results were found between control and lithium-pilocarpine epileptic rats in extracts from hippocampal formation, cerebellum and cortex (Melo *et al.* 2005) and in lithium-pilocarpine epileptic mice (Smeland *et al.* 2013).

Although statistical significance was not reached, judging from the amounts, CRS appeared to improve glucose utilization in the CRS-TLE and CRS-ALE rats.

5.2. Glutathione, Taurine and NAA

The amounts of glutamate, aspartate, glutathione, taurine (obtained from HPLC) and NAA (obtained from ^1H NMR spectroscopy) were smaller in amounts in amygdala, entorhinal and piriform cortices in the UT-TLE group compared to the control group (see Fig. 8; Table 2). The smaller amounts of these metabolites in TLE is associated with neuronal loss and/or mitochondrial dysfunction as it has been reported in previous other studies in animal models of TLE (Melo *et al.* 2005).

Taurine is believed to exert several physiological effects in the brain. It is a powerful agent in regulating and reducing calcium homeostasis in neurons and hence protects against neuronal death and excitotoxicity (Chen *et al.* 2001). Taurine lowers the flux of calcium into the cytosol and as a result reduces intracellular calcium concentration during neuronal activation (for review see (Foos & Wu 2002)). Lithium-pilocarpine administration in rats caused a reduction in taurine level and made neurons more vulnerable to cell damaging insults. Carisbamate treatment however was able to prevent the reduction of taurine in the CRS-TLE and CRS-TLE groups.

During oxidative metabolism, ROS are continuously produced in the brain. However, the body's antioxidant system is able to scavenge free radicals. Glutathione is a major antioxidant in the brain that protects cells from oxidative stress (for reviews see (Dringen 2000, Schulz *et al.* 2000, Aoyama *et al.* 2008)). However, during neurological disorders, the balance between the generation of ROS and antioxidative processes is disturbed. Increased production of ROS and reduced antioxidant capability of cells leads to oxidative stress and hampering of cellular functions. Studies in patients with epilepsy and in rat models of epilepsy have shown widespread impairment of the glutathione system (Mueller *et al.* 2001). In our study, lithium-pilocarpine administration has led to a significant reduction in the levels of glutathione in the UT-TLE rats and treatment with CRS kept glutathione levels from dropping in both the CRS-TLE and CRS-ALE groups. This is in agreement with a previous report in the same model (Melo *et al.* 2005).

NAA (N-acetylaspartate) is predominantly found in neurons but not in glial cells (Moffett *et al.* 1991, Urenjak *et al.* 1992). It is considered a marker for neuronal viability and cellular dysfunction (Moffett *et al.* 1991). NAA is reduced when there is

neuronal loss and/or metabolic dysfunction. In this study, the level of this metabolite was significantly reduced in the UT-TLE group compared to the control rats. Treatment with CRS was able to prevent the decrease in NAA levels in both the CRS-TLE and CRS-TLE groups. The level of NAA in the CRS-ALE group was higher than that in the CRS-TLE group and was similar to the control rats. Previous studies have also reported reduced levels of NAA in limbic structures (hippocampal formation, entorhinal and piriform cortex) in the kainate model of epilepsy in rats (Alvestad *et al.* 2008), in the hippocampal formation in the pilocarpine model in mice (Smeland *et al.* 2013) and in hippocampal formation, cortex and cerebellum in rats (Melo *et al.* 2005).

5.3. Effects of carisbamate on taurine, glutathione and NAA

In our study, treatment with CRS prevented the drop in the levels of taurine, glutathione and NAA compared to the UT-TLE rats. CRS protected neurons from death. This in agreement with a previous study that showed the disease modifying and neuroprotective potential of CRS (Francois *et al.* 2011). CRS-ALE rats showed higher NAA level compared to the CRS-TLE rats. This might mean either more protection and/or improved mitochondrial metabolism in the CRS-ALE rats compared to the CRS-TLE rats.

5.4. TCA cycle related metabolites

5.4.1. Metabolites derived from [1-¹³C]glucose

Neuronal metabolism

[4-¹³C]glutamate, [4-¹³C]glutamine and [2-¹³C]GABA are derived from [1-¹³C]glucose in the first turn of the TCA cycle (see Fig. 7). The amounts of [4-¹³C]glutamate and total amounts of glutamate were reduced in the limbic structures of the UT-TLE rats (see Table 3). The % ¹³C enrichment with [4-¹³C]glutamate which is the percentage of [4-¹³C]glutamate relative to the total amount of glutamate was unchanged in the UT-TLE rats compared to the control rats (see Table 5). The reason for the unchanged % ¹³C enrichment might be because both the ¹³C labeled amount and the total glutamate were reduced. Most of the glutamate is located in neurons (Ottersen & Storm-Mathisen 1985) and changes in the amount of labeled and total glutamate shows alterations in neuronal metabolism. A decrease in labeling of glutamate and a concomitant reduction in total glutamate might show either neuronal loss and/or mitochondrial metabolic dysfunction. A reduction in NAA's amount might also show

neuronal loss and mitochondrial dysfunction of the remaining neurons (Moffett *et al.* 1991). Glutathione was also reduced which might also show neuronal loss. However, studies have shown that there is a weak association between neuronal loss and the amount of metabolites including NAA, glutamate, glutamine and GABA. In one study, it was found that the mean level of a metabolite in hippocampus while less than 30% of neurons survived was similar to the level at which more than 70% of neurons survived (Petroff *et al.* 2002b). However, a combination of both neuronal death and neuronal metabolic dysfunction such as changes in transporters, decreased activity in the TCA cycle, and/or various mitochondrial enzymes could be the reason for decreased labeling of glutamate. This is in line with previous reports that showed decreased glutamate levels in animal models of epilepsy (Melo *et al.* 2005, Alvestad *et al.* 2008).

Glutamate is released from neurons and taken up by astrocytes. Astrocytes then synthesize glutamine by the action of GS. So, a reduction in [4-¹³C]glutamate leads to a reduction in [4-¹³C]glutamine since approximately 40% of the [4-¹³C]glutamine formed from [1-¹³C]glucose is derived from glutamate released from neurons (Hassel *et al.* 1997). Total glutamate was reduced, while the total glutamine remain unchanged in the UT-TLE rats. Previous studies have also shown the same results in lithium-pilocarpine rats (Melo *et al.* 2005), pilocarpine mice (Smeland *et al.* 2013) and the kainate models of TLE (Alvestad *et al.* 2008). [4-¹³C]glutamine was reduced in the UT-TLE rats while the total amount was unchanged. The % ¹³C enrichment with [4-¹³C]glutamine was therefore reduced. The decrease in [4-¹³C]glutamine labeling could be either due to less transfer of [4-¹³C]glutamate from neurons to astrocytes or a decreased synthesis of glutamine from glutamate in astrocytes. GS is the enzyme that synthesizes glutamine and its activity was found to be reduced in animal models of epilepsy (Eid *et al.* 2004, Bidmon *et al.* 2008, Hammer *et al.* 2008). The ratio for the neuron to astrocyte transfer was also lowered for the UT-TLE rats which shows reduced transfer of [4-¹³C]glutamate from neurons. This is also evidenced by the unchanged total amount of glutamine in spite of gliosis, an epileptogenic process which occurs in the chronic phase of the lithium-pilocarpine model of TLE (Roch *et al.* 2002). In general, the decreased labeling in [4-¹³C]glutamate and [4-¹³C]glutamine shows a reduced neuronal metabolism in the UT-TLE rats.

GABA labeling and total amount were unchanged in the UT-TLE rats similar with other studies in the lithium-pilocarpine model of TLE in hippocampal formation and cortex (Melo *et al.* 2005) and the kainate induced TLE in hippocampal formation, entorhinal cortex and neocortex (Alvestad *et al.* 2008). Although increased death of GABAergic interneurons was found in the lithium-pilocarpine model of TLE, the activity of GAD (GABA synthesizing enzyme) in the remaining neurons was increased (Andre *et al.* 2001, Silva *et al.* 2002). The decrease in GABA due to loss of interneurons might have been balanced by an increase in the activity of GAD which led to unchanged GABA labeling in the UT-TLE rats.

5.4.2. Effects of carisbamate on neuronal metabolism

CRS treatment was able to keep the amount of glutamate and aspartate from dropping in the CRS-TLE and CRS-ALE rats. In the CRS-ALE rats, the levels of glutamate, glutamine and aspartate were at similar levels to control rats. CRS treatment also increased the level of glutamine and aspartate in the CRS-TLE rats compared to the UT-TLE rats and the level of glutamine was even higher than in the control rats. Treatment with CRS increased labeling of [4-¹³C]glutamate and [4-¹³C]glutamine in the CRS-TLE and CRS-ALE rats. The % ¹³C enrichment with [4-¹³C]glutamate for the CRS treated rats was unchanged. The % enrichment with [4-¹³C]glutamine was increased in the CRS-TLE rats compared to the UT-TLE rats and although not significant it appears increased in the CRS-ALE rats. Increased % enrichment with [4-¹³C]glutamine despite an unchanged total glutamine shows either improved transfer of [4-¹³C]glutamate from neurons to astrocytes or increased activity of GS following treatment with CRS. This is also evidenced by an increased neuron to astrocyte transfer ratio in the CRS-TLE and CRS-TLE rats compared with the UT-TLE rats. However, CRS didn't show effects on GABAergic neurons. In general, these results show that lithium-pilocarpine causes neuronal death and/or mitochondrial metabolic disturbances in rats. However, CRS was able to protect neurons and improve neuronal metabolism following lithium-pilocarpine administration.

5.4.3. Metabolites derived from [1,2-¹³C]acetate

Astrocytic metabolism

Astrocytic metabolism can be assessed by injecting [1,2-¹³C]acetate which is preferentially metabolized in astrocytes (Waniewski & Martin 1998). In astrocytes,

[1,2-¹³C]acetate can be converted into [4,5-¹³C]glutamate. [4,5-¹³C]glutamate is then converted into [4,5-¹³C]glutamine which can be transported into neurons and can be converted into [4,5-¹³C]glutamate in glutamatergic and GABAergic neurons (see Fig. 7). The labeling of [4,5-¹³C]glutamate from [1,2-¹³C]acetate was reduced in the UT-TLE rats compared to the control rats while in [4,5-¹³C]glutamine, the change didn't reach statistical significance ($p=0.07$) though the trend is towards a decrease. The % ¹³C enrichment with [4,5-¹³C]glutamate in the UT-TLE rats was unchanged as there was decreased labeling and decreased total glutamate. The % ¹³C enrichment with [4,5-¹³C]glutamine was unchanged in spite of decreased amount in [4,5-¹³C]glutamine ($p=0.07$) and unchanged total glutamine in the UT-TLE rats. Most of the [4,5-¹³C]glutamate in neurons is from [4,5-¹³C]glutamine in astrocytes. Therefore, the decrease in [4,5-¹³C]glutamate is due to decreased transfer of [4,5-¹³C]glutamine from astrocytes to neurons as a result of either decreased levels of [4,5-¹³C]glutamine that could be transferred to neurons or a disturbance with the transfer of glutamine. However, the transfer ratio of [4,5-¹³C]glutamine from astrocytes into neurons was unchanged (but with 20% reduction) (see Table 6). In addition, there was a decrease in labeling of [4-¹³C]glutamine in the UT-TLE rats, and approximately 60% of [4-¹³C]glutamine is obtained from the astrocytic TCA cycle (Hassel *et al.* 1997). These results thus show that astrocytes might be affected in the limbic structures leading to a decrease in mitochondrial astrocytic metabolism. Reduced astrocytic metabolism has also been shown in the kainate models of TLE in hippocampal formation and neocortex (Alvestad *et al.* 2011). In contrast to this study, astrocytes were not affected in the hippocampal formation, cerebral cortex and neocortex in the lithium-pilocarpine model of TLE (Melo *et al.* 2005).

Labeling and the % enrichment with [1,2-¹³C]GABA were unchanged ($p=0.076$) in the UT-TLE rats although the trend is towards a decrease (approximately 38% and 30% reduction respectively). The decrease in the levels of [1,2-¹³C]GABA could be due to neuronal loss. Loss of GABAergic interneurons and other alterations and hence reduced inhibition is one of the factors in epileptogenesis in the lithium-pilocarpine model in rats (Andre *et al.* 2001) and in mouse models of TLE (Bouilleret *et al.* 2000).

5.4.4. Effects of carisbamate on astrocytic metabolism

CRS treatment didn't prevent the level of [4,5-¹³C]glutamate from dropping. However treatment with CRS led to a significant increase in [4,5-¹³C]glutamine in the CRS-ALE rats. The % ¹³C enrichment with [4,5-¹³C]glutamate was reduced in the CRS-ALE and CRS-TLE rats. This is due to an increase in total glutamate caused by CRS treatment. However, the % ¹³C enrichment with [4,5-¹³C]glutamine in the CRS-ALE rats after CRS treatment was unchanged because there was an increase in both [4,5-¹³C]glutamine and total glutamine. It seems CRS improved astrocytic metabolism in the CRS-ALE rats which was shown by increased glutamine content while this was not affected in the CRS-TLE rats. CRS treated rats had lower % ¹³C enrichment with [1,2-¹³C]GABA compared to the control rats. The astrocyte to neuron transfer ratio for GABA was unchanged. CRS didn't show an effect on GABAergic neurons. It might be due to neuronal loss in the limbic structures.

The acetate/glucose utilization ratio, which is the proportion of ¹³C label derived from acetate relative to that from glucose, was increased in glutamate for the UT-TLE rats (p=0.058) (but not statistically significant) compared to controls and decreased in the CRS-TLE and CRS-ALE rats compared to the UT-TLE rats. The ratio is also increased for glutamine in the UT-TLE rats. Increased use of acetate relative to glucose in the UT-TLE rats might be either due to astrogliosis or neuronal death. Loss of neurons in the limbic structures and proliferation of astroglial cells has been observed in the lithium-pilocarpine animal model of TLE (Groticke *et al.* 2007). However, treatment with CRS shifts the balance and caused increased use of glucose as a precursor probably due to improved neuronal metabolism and neuroprotection. In general, it seems that most of CRS's effects are related to improvement in neuronal metabolism rather than astrocytic metabolism.

5.5. Differences between the CRS-TLE and CRS-ALE rats

There were some differences observed between the CRS-TLE and CRS-ALE rats. The first difference observed was increased levels of total glutamine in the CRS-ALE rats. Glutamine content was even higher than in the control rats. [4,5-¹³C]glutamine was also found to be higher in the CRS-ALE rats. Downregulation of GS, the enzyme which synthesizes glutamine in hippocampus with severe neuronal loss has been observed in temporal lobe epilepsy patients (van der Hel *et al.* 2005). A deficiency in

GS is thought to be the basis for extracellular glutamate increase and hence seizure generation in TLE (Eid *et al.* 2004). CRS treatment probably increased glutamine level in the CRS-ALE rats by improving GS activity or by preventing neuronal loss. As glutamine is synthesized from glutamate, preventing glutamatergic neuronal loss might also be the reason for increased glutamine production. CRS has been shown to be a strong neuroprotective agent (Francois *et al.* 2011). Another difference observed between the CRS-TLE and CRS-ALE groups was the level of NAA. NAA is a marker of neuronal viability and the level of NAA was higher in the CRS-ALE rats compared to the CRS-TLE rats. This might show more neuroprotection in the CRS-ALE rats than in CRS-TLE rats. Aspartate levels were also higher in the CRS-ALE rats which might also be related to more neuroprotection in the CRS-ALE group. PC activity seen in the formation of glutamate was reduced in the CRS-TLE rats; however it was similar to the control rats following CRS treatment in the CRS-ALE group. The anaplerotic PC might be functioning better in the CRS-ALE than in the CRS-TLE rats.

6. Conclusion

We showed that alterations occur in neuronal and astrocytic metabolism in limbic structures of rats with lithium-pilocarpine induced temporal lobe epilepsy. Lithium-pilocarpine administration caused neuronal loss and/or metabolic dysfunction. Glucose metabolism was also impaired and the neuronal- astrocytic metabolic interaction was affected. CRS altered the damage caused by seizures induced by lithium-pilocarpine. It improved neuronal metabolism and prevented neuronal death which was shown by increased levels of metabolites such as glutathione, NAA and taurine. CRS-ALE rats showed increased glutamine content and more neuroprotection compared to the CRS-TLE rats.

7. References

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8. Appendix: Methanol-Chloroform extraction

Method	Methanol-chloroform double extraction
Written by	Based on method from Lindy Rae
Original	
Revisions	Mussie Ghezu/ Lars Evje/Ursula Sonnewald
Date	04.09.2012

MOST STEPS HAVE TO BE DONE IN THE FUME HOOD, AND USE NITRILE GLOVES

USE ONLY TUBES MADE OF POLYPROPYLENE AND NOT E.G. POLYSTYRENE

This protocol is revised to consider for the way samples were sent from Strasburg (France).

Chemicals

List of chemicals used in the experiment

Name	Supplier	Catalog number	CAS no.	Where
Chloroform (CHCl ₃)	Sigma	319988	67-66-3	Giftskap nederst
Methanol (CH ₃ OH)	Lab-Scan	C17C11X	67-56-1	K13
L-2-Aminobutyric acid (α -ABA, C ₄ H ₉ NO ₂)	Fluka	07200	1492-24-6	B36
Water has to be distilled or purified				

Equipment

- Eppendorf Centrifuge 5702R (Germany)
- Elga UltraAnalytic water purification system (Great Britain)
- Grant QBT4 block heater (Great Britain)
- Mettler Toledo XP-205 balance (USA)
- Sonics VCX750 with **stepped** microtip 250 μL – 10 mL (catalog no. 630-0422) ultrasonic processor (USA)

Extraction procedure

The procedure is for up to 150 mg tissue. a-ABA is an internal standard for HPLC

Turn on the centrifuge and set the temperature at 4 °C

1. Make sure you have prepared three sets of test tubes for each sample and label them appropriately. A 5 ml tube (tube 1), another 5 ml tube (tube 2) and a 15 ml tube (tube 3)
2. Use a small spatula with flat ends to scrape brain tissue from eppendorf tube (they were delivered in 0.6 ml eppendorf tubes) into Tube 1
3. Add 300 μL methanol (2 times the tissue weight in mg) into the eppendorf tube. Cut off the tip of the microtip used to transfer the methanol into the eppendorf and use it to scrape any remaining tissue in the tube and mix the tissue in the methanol.
4. Transfer the mixture in the eppendorf tube into Tube 1 using the cut off microtip.
5. Add about 50 μL methanol to the eppendorf tube to rinse any remaining tissue
6. Using the cut off microtip scrape any remaining tissue in the eppendorf tube, mix it well and transfer it to Tube 1.
- 7. Add 200 μL ¹ 250 μM a-ABA to Tube 1**

¹ The amount of a-ABA depends on the volume used to dissolve the sample after lyophilization in step 18. The final concentration of a-ABA in the HPLC sample should be 25 μM

8. Sonicate the samples on ice – program 9 (Time 0:00:10; Puls On 01; Puls Off 04; Amplitude 30 %)
9. Look at the sample. If necessary repeat step 4 after waiting approximately 1 minute.
10. Flush the probe with 150 μ L water into **tube 1**
NB: For steps 1 to 10 one sample is taken at a time while the other samples are kept in the -80.
11. **All work with chloroform in fume hood:** Add 200 μ L chloroform (1.5 times the tissue weight in mg) to each tube.
12. Vortex each tube until you get a milky appearance
13. Centrifuge at 4 °C at 4400 rpm (\approx 3000 g) for 15 minutes
14. Use a pipette to transfer the top layer (methanol/water phase) to a new tube (**tube 2, use 5 mL tubes for 150 mg tissue**). Store the tube on ice
15. To **tube 1** add 400 μ L methanol (3 times the tissue weight in mg), add 300 μ L water (2 times the tissue weight in mg) and 100 μ L chloroform
16. Vortex
17. Repeat steps 13 and 14
18. To **tube 1** add 200 μ L methanol (1.5 times the tissue weight in mg) and 150 μ L water (1 times the tissue weight in mg) and vortex.
19. Repeat step 13 and 14
20. Add 200 μ L chloroform and 1500 μ L water to **tube 2 (see comment below)**
21. Vortex
22. Centrifuge at 4 °C at 4400 rpm for 15 minutes and transfer the top layer to new 15 mL tube (**tube 3**)
23. Add 500 μ L water and 100 μ L methanol to **tube 2**
24. Repeat step 21 and 22
25. Once frozen (-80 °C) evaporate the methanol/water from **tube 3** using the lyophilizer.
Tube 3 is used for e.g. NMR, GC-MS and HPLC and can be stored at -20 °C.

26. If you need to know the protein concentration use **tube 1** which is left in a fume hood to evaporate the chloroform. Then dissolve the pellet in NaOH and perform protein determination or store in freezer for other analyses.

Tube 2 and the pipette tips used after step 6 must be left in a fume hood to evaporate the chloroform – throw next day.

Comment:

The metanol:water phase in tube 2 will contain lipids. The addition of water in step 16 is done in order to remove the lipids. However, the amount of water in the previous version (protocol from 20120219) is probably not sufficient to remove all the lipids. Therefore the volume of water in step 16 has been increased from 500 μL to 1500 μL .