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^{13}C NMRS of animal models of schizophrenia

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
Faculty of Medicine
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Abbreviations

AAAD	Aromatic amino acid decarboxylase
AAT	Aspartate aminotransferase
Acetyl CoA	Acetyl Coenzyme A
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionsyre
ATP	Adenosine triphosphate
B_0	External applied magnetic field
d1	Relaxation delay
D1/D2	Dopamine receptors
DOPAC	3,4-dihydroxyphenylacetic acid
D_2O	Deuterated water
EAAC1	Excitatory amino acid transporters
FCR	Frontal cortex plus cingulate and retrosplenial cortices
FCX	Frontal cortex
FID	Free induction decay
GABA	γ-amino butyric acid
GABA-T	GABA transporters
GAD	Glutamic acid decarboxylase
GAT	GABA transporters
GC-MS	Gas chromatography- mass spectroscopy
GDH	Glutamate dehydrogenase
GLAST	Glutamate aspartate transporter
GLT	Glutamate transporters
GLUT	Glucose transporters
GS	Glutamine synthetase
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
i.p.	Intraperiotneal
LDH	Lactate dehydrogenase
L-DOPA	3,4-dihydroxy-L-phenylalanine
m/z	Mass to charge ratio
MCT	Monocarbocxylate transporters
MK-801	Dizocilpine
NAA	N-acetyl aspartate
NAc	Nucleus accumbens
NAD+	Nicotinamide adenine dinucleotide, oxidated form
NADH	Nicotinamide adenine dinucleotide, reduced form
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser Effect
PAG	Phosphate-activated glutaminase
PC	Pyruvate carboxylase
PCA	Percloric acid
PCP	Phencyclidine
PDH	Pyruvate dehydrogenase
PPI	Pre pulse inhibition
PPM	Parts per million
PTCX	Parietal and temporal cortex
RF	Radio frequency
SSADH	Succenic semialdehyd dehydrogenase
T_1	The longitudinal relaxation time

TCA cycle	Tricarboxylic acid cycle
TE	Temporal lobe
α-KG	α-ketoglutarate

Summary

Altered brain metabolism is implicated in several brain disorders such as schizophrenia. Insights into underlying mechanisms and how they are altered could help find new treatment strategies.

Animal models serve as tools to mimic human diseases. However, recreating the "normal" course of human disease in animals is difficult. Nevertheless, animal models have shown to be useful in providing knowledge about pathological processes. In this thesis several animal models of brain disorders were used. We used both pharmacological interventions mimicking one feature of schizophrenia, and a gene knock out model aimed at elucidating the role of GAD_{65} in disease. GABA metabolism is believed to be altered in several brain disorders. Two different protocols for studying the effect of MK-801 were used to mimic schizophrenia, repeated low dose (0.1 mg/kg) MK-801 and repeated high dose injections (0.5 mg/kg) MK-801 respectively. These models were used to gain knowledge about how altered neurotransmitter homeostasis possibly can lead to psychiatric disease.

In paper I, repeated low dose MK-801 injections caused hypermetabolism of glucose and increased glutamatergic activity in the temporal areas only. Thus, it appears that this model does not show the same pattern as seen in patients with schizophrenia but rather mimics the toxic effects of MK-801 possibly caused by increased glutamate release into the synaptic cleft. Repeated injections of high doses of MK-801 (paper II) led to hypometabolism of glucose. It was further shown that perturbation of NMDA receptor function in the model of repeated injections of MK-801 caused changes not only in the glutamatergic and GABAergic systems, but also in that of dopamine. Changes were most pronounced in the frontal cortex (FCX) in analogy with the human condition. In paper II, repeated injection of high doses of MK-801 resulted in increased amounts of glutamate. However, reduced ^{13}C labelling was observed in the same study, which might indicate a transition to reduced glutamate metabolism, and glutamate amounts seen in patients with chronic schizophrenia. In paper III, also using injection of repeated high doses of MK-801, we found similar results as in paper II, with reduced ^{13}C labelling in glutamate and glutamine. Further we found reduced ^{13}C labelling in GABA, lactate and NAA implying neuronal hypometabolism.

In paper IV studying GAD_{65} knockout mice, labelling from glucose was dramatically decreased in lactate and alanine reflecting attenuated glycolysis. In concurrence with this TCA cycle activity, was decreased in the GAD_{65} knockout animals. Consequently, decreased ^{13}C labelling in GABA was observed, implying neuronal hypometabolism.

Patients with schizophrenia constitute a heterogeneous group with a large variety of symptoms and it is likely that the underlying causes of psychosis are not always induced by the same mechanisms. Still, it is of great interest that blocking of the NMDA receptor using repeated injections of high doses of MK-801 caused neuronal hypometabolism as found in the GAD_{65} knockout model. Hypometabolism in FCX is a common finding in patients with schizophrenia.

Sammendrag

En rekke lidelser som for eksempel schizofreni og epilepsi er forårsaket av forstyrrelser i hjernemtabolismen. Patofysiologien og de bakenforliggende mekanismene er dårlig klarlagt, dermed fungerer dagens behandlingsmetoder i varierende grad. Å kartlegge de metabolske prosessene og hvordan de er påvirket i disse lidelsene vil derfor kunne gi ny viten som videre kan benyttes til å finne nye behandlings strategier.

Dyremodeller er et nyttig verktøy når man studerer lidelser som schizofreni, men å gjenskape et naturlig sykdomsforløp er vanskelig. Til tross for dette har dyremodeller vist seg å ha stor nytteverdi ved at de gir oss muligheten til å studere patologien i en rekke lidelser i detalj. I denne avhandlingen ble flere dyremodeller benyttet. Vi benyttet både farmakologiske modeller, som er nyttige for å etterligne forløpet i schizofreni og genetisk modifiserte modeller. Sistnevnte er en genetisk "knockout" modell som er nyttig for å belyse rollen av GAD_{65} i metabolismen av GABA, da metabolismen av GABA antas å være endret i schizofreni. I de to studiene hvor farmakologis påvirkning ble benyttet, ble to ulike doserings protokoller fulgt, en med gjentatte injeksjoner lav dose (0,1 mg / kg) MK-801 og en med gjentatte injeksjoner høy dose (0,5 mg / kg) MK-801. Disse modellene ble benyttet for å få kunnskap om hvordan en endret neurotransmitter homeostase kan føre til psykiatriske lidelser.

I artikkel I benyttet vi gjentatte injeksjoner med lav dose MK-801, vi observerte at denne injeksjons protokoll forårsaket en hypermetabolisme av glukose og økt glutamaterg aktivitet i de temporale områdene. Dermed ser det ut til at denne modellen ikke viser det samme mønster som i pasienter med schizofreni, men heller modellerer den toksiske effekten av MK-801, antageligvis på grunn av økt glutamat frigjøring i den synaptiske kløften. Derimot førte gjentatte injeksjoner av høy dose av MK-801 (artikkel II) til hypometabolisme av glukose. Vi viste videre at forstyrrelse og blokkering av NMDA reseptorfunksjon forårsaket endringer ikke bare i de glutamaterge og GABAerge systemer, men også de dopaminerge. I samsvar med funn fra klinikken fant også vi de største forandringene i fremre deler av cortex (FCX). I artikkel II, så vi at gjentatte injeksjoner av høy dose MK-801 resulterte i en økt glutamat konsentrasjon. Imidlertid observerte vi en redusert innmerkning av ^{13}C i glutamat i samme studie. Dette kan indikere en overgang til redusert glutamat metabolisme og følgelig en redusert mengde av glutamat, som man har funnet i kliniske studier av. I artikkel III, benyttet

vi også gjentatte injeksjoner med høy dose MK-801. I denne studien ble det funnet en redusert innmerkning av ^{13}C glutamat og glutamin. Videre fant vi en redusert innmerkning av ^{13}C i GABA, laktat og NAA, dette indikerer en neuronal hypometabolisme.

I artikkel IV, hvor vi benyttet en genetisk modifisert musemodell, en GAD_{65} knockout mus, observerte vi en redusert innmerkning laktat og alanin, dette gjenspeiler en redusert glykolyse. I samsvar med dette observerte vi også en redusert TCA syklus aktivitet i GAD_{65} knockout musene. En redusert ^{13}C innmerkning i GABA ble også funnet. Disse funnene med en redusert ^{13}C innmerkning i metabolittene indikerer en neuronal hypometabolisme.

Pasienter med schizofreni utgjør en heterogen gruppe med varierte symptomer. Det er sannsynlig at de bakenforliggende årsakene til psykoser hos de rammede ikke alltid er forårsaket av de samme mekanismene. Likevel er det bemerkelsesverdig at en blokkering av NMDA reseptoren ved gjentatte injeksjoner av høye doser MK-801 forårsaket lignende funn som i GAD_{65} knockout musemodellen, altså en neuronal hypometabolisme. En slik hypometabolisme i FCX er et vanlig funn hos pasienter med schizofreni.

List of publications

The thesis is based on the following publications

Paper I

Eyjolfsson E.M, Brenner E, Kondziella D, Sonnewald D. *Repeated injection of MK801: an animal model of schizophrenia?* Neurochem Int 2006.

Paper II

Kondziella D, Brenner D, **Eyjolfsson E.M**, Markinhuhta K.R, Carlsson M.L, Sonnewald U. *Glial-Neuronal Interactions are Impaired in the Schizophrenia Model of Repeated MK-801 Exposure.* Neuropsychopharmacology 2006.

Paper III

Eyjolfsson E.M, Nilsen L.H, Kondziella D. Brenner E. Sonnewald U, Håberg A. *Altered Metabolism of Amino Acid Neurotransmitters and Dopamine in the Repeated MK-801 Animal Model of Schizophrenia.* Submitted, 2009.

Paper IV

Walls A.B, **Eyjolfsson E.M,** Vestergaard H.T, Hansen S.L., Schousboe I,.Sonnewald U, Schousboe A, Wagepetersen H.S. *Knockout of GAD_{65} has major impact on synthesis from astrocyte-derived glutamine of vesicular GABA acting extrasynaptically.* Manuscript, 2009.
**Shared first authorship*

Following papers are not included in this thesis.

Kondziella D, **Eyjolfsson E.M**, Sæther O, Sonnewald U, Risa Ø.
Gray matter metabolism in acute and chronic hydrocephalus. Neuroscience, 2009.

Alvestad S, Hammer J, **Eyjolfsson E.M**, Qu H, Ottersen O.P, Sonnewald U. *Limbic structures show altered glial-neuronal metabolism in the chronic phase of kainate induced epilepsy*. Neurochem Res. 2008.

Kondziella D, Brenner E, **Eyjolfsson E.M**, Sonnewald E. *How do glial-neuronal interactions fit into current neurotransmitter hypotheses of schizophrenia?* Neurochem Int. 2007.

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^{13}C NMRS of animal models of schizophrenia.

1. Introduction

Altered brain metabolism is implicated in several brain disorders such as schizophrenia and epilepsy. Insights into underlying mechanisms and how they are altered could help find new treatment strategies. This thesis will focus on animal models of schizophrenia.

1.1 Brain cells (Raine, 2006)

The brain consists of two main types of brain cells; **neurons** and **glia**.

Neurons are the main signal transmitters that constitute the main components of the brain and the nervous system. Neurons, unlike other cells in the body, have anatomical and functional specializations for transmission of chemical and electrical signals that make them essential for successful transmission of signals over short and long distances. Neurons have a large variety of dendritic and axonal outgrowths, and highly specialized structures at the end of the axon, called synapses. The characteristics of these structures vary depending on their localization in the body and the species studied. The brain consists of another main type of cells, the **glia**. Unlike neurons, glia cells have no conventional synaptic contacts. In the brain the glia and neurons are intimate partners, thus glia may participate in the dysfunction seen in different brain disorders such as schizophrenia and epilepsy (Kondziella et al., 2006; Melo et al., 2006). Glia cells can further be divided into astrocytes, oligodendrocyttes, and microglia. In this thesis interactions between neurons and astrocytes are in focus. Astrocytes constitute the majority of the glia in the brain and have extensive contacts to other brain cells. Numbers of astrocytes per neuron increases in line with the complexity of the brain (Nedergaard et al., 2003). Astrocytes participate in the regulation of neurotransmitters homeostasis, and play an important part in re-uptake of glutamate from the synaptic cleft. Astrocytes also have an active role in the synthesis of

precursors used by neuronal synthesis of glutamate and GABA (Sonnewald et al., 1993; Waagepetersen et al., 2001b).

2. Brain metabolism and the TCA cycle

The brain is very energy demanding compared to its size, considering the brain constitutes only 2% of a humans total body weight it requires ~20% of the total energy consumption. It has been proposed that ~75% of the energy used by the brain is used for signal processes such as action potentials, glutamate cycling and so on (Attwell and Laughlin, 2001). To maintain normal brain function the brain needs a supply of energy substrates like glucose. Glucose is the most important energy substrate in the brain and is included in a number of possesses. Through glycolysis and the tricaboxylic acide cycle (TCA cycle) the metabolism of glucose provides necessary building blocks for synthesis of neurotransmitters. Brain metabolism and synthesis of neurotransmitters is divided between different compartments in the brain (Waagepetersen et al., 2001a; Waagepetersen et al., 2003). This thesis focuses on the compartmentation between neurons and astrocytes.

2.1 Metabolism of glucose

Both astrocytes and neurons metabolise glucose. Glucose is transported over the blood brain barrier and transported from the extracellular space into the intracellular space by special glucose transporters. Several different isoforms of glucose transporters have been identified (ranging from GLUT1 to GLUT4), these have different locations in the body and brain (Danbolt, 2001). GLUT3 is the primary glucose transporter in neurons, while GLUT1 is mainly found in astrocytes (Maher, 1995; Danbolt, 2001). Once within the intracellular space, glucose is metabolised by several steps in the glycolysis to pyruvate. One glucose molecule yields two molecules of pyruvate and two molecules of ATP. Thereafter pyruvate

is transported over the mitochondrial membrane by mitochondrial carriers. Once pyruvate has entered the mitochondria, as seen in figure 1, pyruvate is metabolised and can enter the TCA cycle either through pyruvate dehydrogenase (PDH) or through pyruvate carboxylase (PC). Via PDH, which is found in both neurons and astrocytes, pyruvate is decarboxylated to acetyl CoA, that enters the TCA cycle and forms citrate, (as seen in figure 1) further providing building blocks for the TCA cycle. The other pathway is through the anapplerotic enzyme PC, an enzyme that is more pronounced in astrocytes (Yu et al., 1983). This pathway transforms pyruvate to oxaloacetate, a metabolite in the TCA cycle. Thus astrocytes and neurons have different ways of providing the TCA cycle with building blocks that can be further used in synthesis of neurotransmitters.

2.2 Metabolism of acetate

Acetate is mainly metabolised by the astrocytes (Cerdan et al., 1990; Hassel et al., 1995; Cruz et al., 2005). Hence acetate can be used to study brain metabolism as more precise metabolic information about glial-neuronal metabolism can be obtained. Acetate is transported into the astocytes by monocarboxylate transporters (MCTs) (Waniewski and Martin, 1998), and by several steps acetate enters the TCA cycle as acetyl CoA.

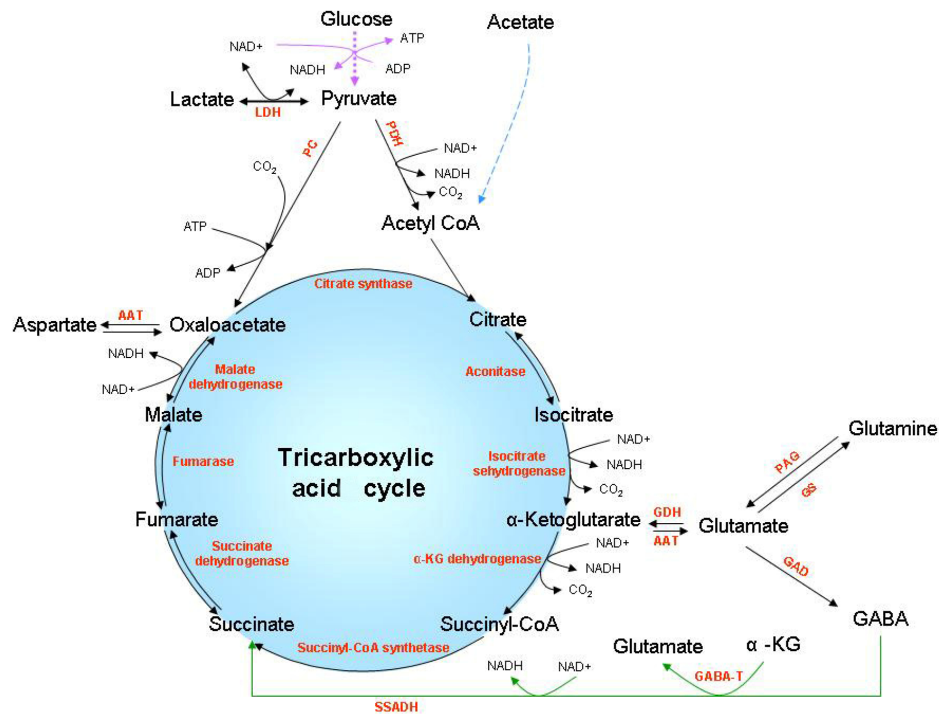


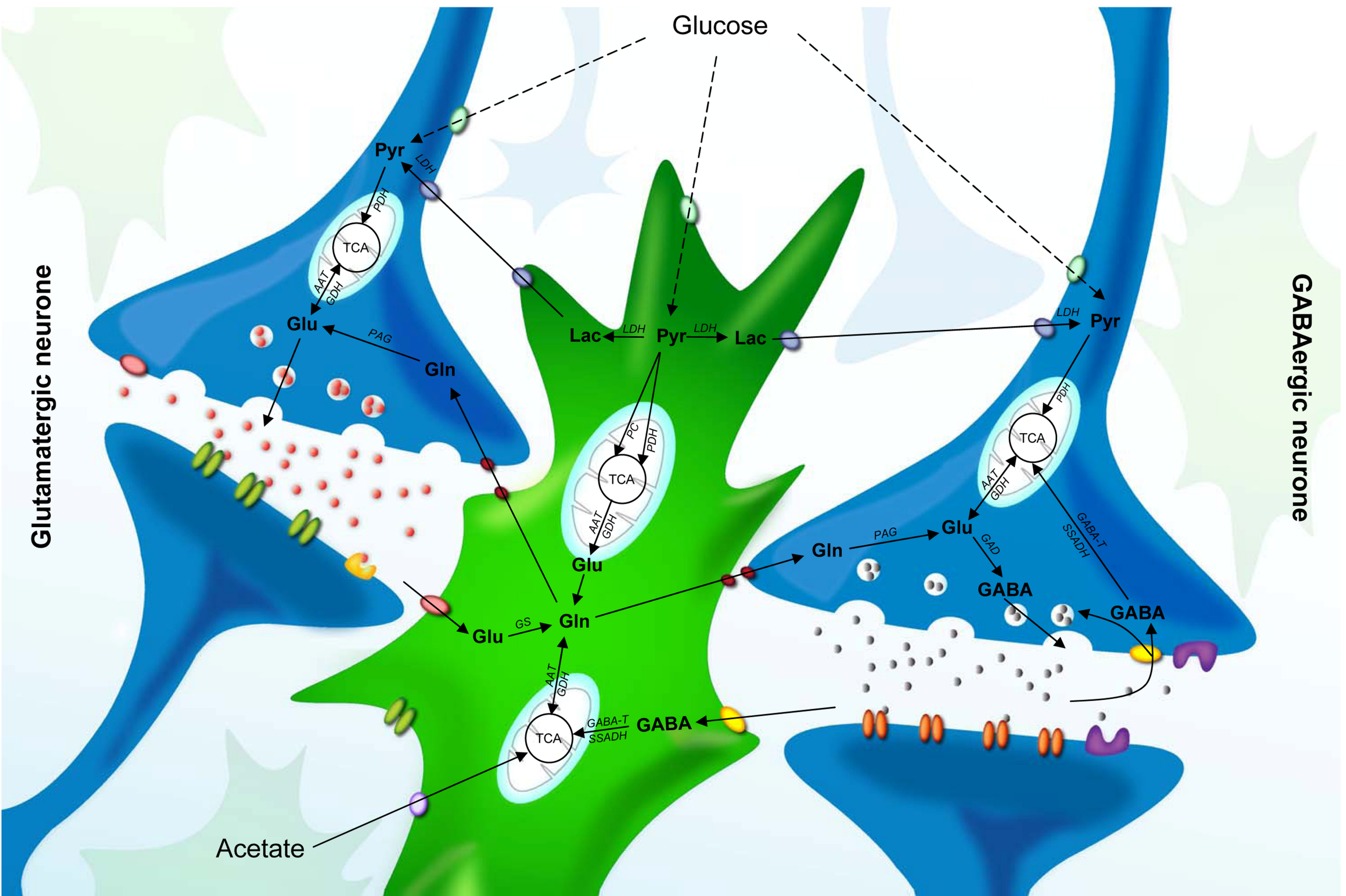
Figure 1. The glycolysis and the TCA cycle. *Glucose is taken up by neurons and astrocytes via specific glucose transporters. Glucose is through several steps in the glycolysis (illustrated with purple arrow) metabolized to pyruvate in the cytocol. Pyruvate is then transported across the mitochondrion membrane and enters the TCA cycle either via PDH as acetyl CoA, or the anaplerotic enzyme PC as oxaloacetate. Illustrated with blu arrow; the parthway of metabolism of acetate in astrocytes. Illustrated with green arrow is the GABA shunt. See text for further explanations. Abbreviations; Lactatedehydrogenase **(LDH)** pyruvate carboxylase **(PC)**, pyruvate dehydrogenase **(PDH),** glutamate dehydrogenase **(GDH),** aspartate amino transferase **(AAT)**, phosphate activated glutaminase **(PAG)**, glutaminase **(GS)**, glutamic acide decarboxylase **(GAD)**, succenic semialdehyd dehydrogenase **(SSADH)**, Gamma-Aminobutyric acid transaminase **(GABA-T)**. Modified from (Berg, 2006).*

3. Neurotransmitter systems

Neurotransmitters are chemical substances interacting at synapses between nerve cells in the nervous system. Here we find both excitatory and inhibitory neurotransmitters. In this thesis three transmitters are of special interest, the aminoacids glutamate and GABA, and monoamines. All of these are involved in most aspects of maintaining brain function and alteration in the neurotransmitter homeostasis may cause severe health impairments (Sonnewald and Kondziella, 2003). These transmitters are synthesised in the presynaptic terminal and stored in vesicles until they are released by mediation of an action potential and influx of Ca^{2+}. After release from the vesicles, the neurotransmitters are either taken up by the neurons or the astrocytes.

3.1 Glutamate

Glutamate is the primary excitatory neurotransmitter in the brain and is involved in many aspects of the functioning brain. Glutamate is a nonessential aminoacid that does not cross the blood brain barrier and must be synthesised by local precursors (Danbolt, 2001). Glutamate is synthesised in a close collaboration between neurons and astrocytes known as the glutamate -glutamine cycle (Figure 2) (Westergaard et al., 1995).



Glucose
Glutamatergic neurone
GABAergic neurone
Pyr
LDH
PDH
TCA
AAT
GDH
Glu
PAG
Gln
Lac
Pyr
Lac
PC
PDH
TCA
AAT
GDH
Glu
GS
Glu
Gln
AAT
GDH
TCA
GABA-T
SSADH
GABA
Acetate
LDH
Pyr
PDH
TCA
AAT
GDH
Glu
PAG
Gln
GAD
GABA
GABA-T
SSADH
GABA

Figure 2: A simplified illustration of the interactions between astrocytes and neurons. *Glucose enters neurons and astrocytes via special glucose transporters (Illustrated as green circles). Once inside the cells glucose is converted to pyruvate via glycolysis. Pyruvate has several fates: it can be converted to lactate by lactate dehydrogenase (LDH), alanine (not shown) or it can be converted to acetyl CoA by the enzyme pyruvate dehydrogenase (PDH). Or, in astrocytes, pyruvate can be converted by pyruvate carboxylase (PC) to oxaloacetate. Lactate is transported from the astrocytes to the neurons via monocarboxylate transporters (MCT) (illustrated as purple circles). Once in the neurons, lactate is converted to pyruvate and enters the TCA cycle. In the glutamatergic neurons pyruvate is condensed with oxaloacetate and after several steps converted to α-ketoglutarate (α -KG) and glutamate via the enzymes aspartate aminotransferase (AAT) or glutamate dehydrogenase (GDH). Glutamate is stored in vesicles and can be released into the synaptic cleft (glutamate illustrated as red dots) and activates glutamate receptors (NMDA illustrated as green, and AMPA illustrated as yellow). Glutamate is then transported from the synaptic cleft, either into the presynaptic neuron, the postsynaptic neuron or into surrounding astrocytes via glutamate transporters (GLT). Inside the astrocytes, glutamate is converted to glutamine, via glutamine synthetase (GS) and transported back to the neurons via glutamine transporters. Glutamate is then converted back to glutamate via phosphate activated glutaminase (PAG), which is either stored in vesicles ready for use in neurotransmission, or it is converted to α-KG via AAT. This transport of glutamate and glutamine between astrocytes and neurons is referred to as the glutamate-glutamine cycle.*

In GABAergic neurons GABA is synthesized from glutamate via the enzyme glutamic acid decarboxylase (GAD). GABA is then stored in vesicles for release into the synaptic cleft (GABA is illustrated as grey dots) activating GABA receptors on the postsynapse. GABA is removed from the synapse by the GABAergic presynaptic neurone or by the astrocytes via GABA transporters (GAT). GABA is then via GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSDAH) converted to succinate which can via the TCA cycle be used for the formation of α-KG and thus glutamate. In astrocytes the same pathway is active. However, glutamate can be converted to glutamine that is transported to the GABAergic neuron for synthesis of glutamate via PAG and further to GABA. This is referred to as GABA-glutamine-glutamate cycle.

Acetate is exclusively taken up into the astrocytes via MCT. Acetate serves as an additional energy substrate for astrocytes and enters the TCA cycle via acetyl CoA providing metabolites as described for glucose above.

3.1.1 The glutamate-glutamine cycle

As seen in figure 1, glutamate can be formed from two precursors, α-KG and glutamine. The neuronal TCA cycle provides precursors to glutamate synthesis in form of α-KG, which can be transaminated by aspartate aminotransferase (AAT) to form glutamate. However glutamate can also be synthesized from glutamine released from the astrocytes (Hassel et al., 1995; Westergaard et al., 1995; Sonnewald and McKenna, 2002). Glutamine is then taken up by glutamatergic neurons and converted to glutamate by the mitochondrial enzyme phosphate activated glutaminase (PAG). This enzyme is more active in the neurons than in the astrocytes (Kvamme et al., 2001). Glutamate is then stored in vesicles and as a response to increased intracellular Ca^{2+} concentration, released in to the synapse. Glutamate exerts its role by acting on glutamate receptors located in the neurons expressing them. The receptors present in the synapse can be either ionotrophic, or metaboptrophic (Purves, 2001). The ionotrophic receptors are divided into three classes, N.methyl-D-aspartate (NMDA) receptor, (which is central in this thesis and will be described in further detail) the α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptor and the kainic acid receptor. Activation of these receptors can lead to depolarisation of the postsynaptic neuron. During glutamate release, the concentration of glutamate in the synapse can rise from 2-5µM, before release, to 50-100 µM after depolarisation. The concentrations of glutamate released from the postsynapse determine the extent of receptor stimuli. It is critical to keep the extracellular concentration of glutamate low to prevent excessive activation of glutamate receptors as well as high amounts of glutamate are toxic. To prevent a toxic effect and to reutilize the released glutamate, extracellular glutamate is removed from the synapse, by uptake mechanisms in the synapse, both presynaptic and postsynaptic (Danbolt, 2001). The neuronal uptake is performed by special transporters located in the synapse; EAAC1 is the most common neuronal transporter. Glutamate can also be removed from the synapse by the astrocytes, GLT1 and GLAST are the most common astrocytic glutamate transporters (Maragakis and Rothstein, 2001). Distribution of the glutamate transporters differ between the different brain areas and between the different cell types. Studies

suggest that the astrocytic reuptake of glutamate is the predominant pathway to inactivate glutamate in the forebrain (Maragakis and Rothstein, 2001). Glutamate taken up by the astrocytes is metabolised to glutamine by the enzyme glutmaine synthetase (GS) for transport back to the neurons and subsequently used for glutamate synthesis (Schousboe, 1981; Waagepetersen et al., 2005). Studies have shown that approximately 40 % of glutamine in astrocytes is derived from neuronal glutamate removed from the synapse by astrocytes (Hassel et al., 1997). The fact that such a large amount of glutamate is taken up by astrocytes and that neurons are incapable to synthesise *de novo* make the neurons dependent on transfer of building blocks from the astrocytes (Hertz et al., 1999; Zwingmann and Leibfritz, 2003). In addition in astrocytes, the anaplerotic enzyme PC is more active and thus capable of replenishing the TCA cycle. This close relationship and transfer of metabolites between astrocytes and neurons is called the glutamate-glutamine cycle (Westergaard et al., 1995).

3.1.2 The glutamate receptors

Several glutamatergic receptors exist; these are divided into two main sub groups, **ionotrophic,** and **metaboptrophic**. The ionotrophic receptors are divided into three classes, the N-methyl-D-aspartate (NMDA) receptor, the α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptor and the kainic acid receptor. Activation of these receptors can lead to depolarisation of the postsynaptic neuron (Purves, 2001).

The NMDA receptor requires binding of both glutamate and glycine together with removal of Mg^{2+} that block the ionchanel to cause a depolarization. Removal of the Mg^{2+} ion is usually caused by activation of AMPA receptors close by. The NMDA channel is permeable to Na^{+}, K^{+} and Ca^{2+}(Waxham, 1999; Purves, 2001).The NMDA receptor is distributed throughout the brain and is involved in several processes in the brain that include perception, motor control, and several cognitive processes. These systems are believed to be disturbed in patients with schizophrenia. Substances acting on the NMDA receptors and the glutamatergic system are widely used in research on schizophrenia (Olney and Farber, 1995; Newcomer et al., 1999; Olney et al., 1999;

Brenner et al., 2005; Eyjolfsson et al., 2006; Kondziella et al., 2006) . In paper I, II and III the function of this receptor was altered using the NMDA antagonist MK-801. The NMDA receptor consists of several subunits; these subunits have varied localizations in the brain (Hassel, 2006) and might have varied potential for binding of different drugs (Coyle, 2004; Perera et al., 2008).

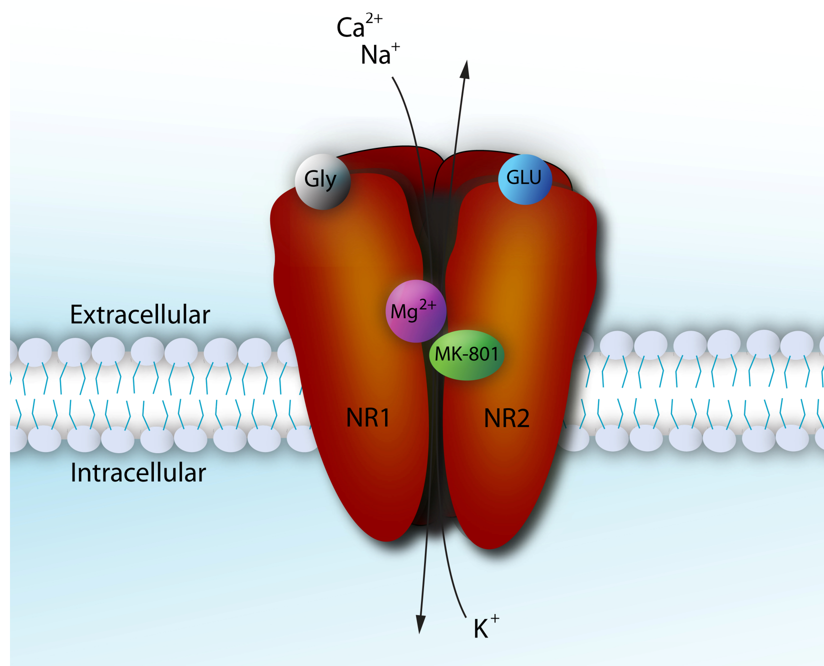


Figure 3: Illustration of the NMDA receptor*. The NMDA receptor is a voltage dependent ion channel. It is activated by glutamate (blue ball) in the presence of glycine (grey ball), this together with membrane activation, induced by nearby AMPA receptors, causes the Mg^{2+} ion (purple ball) to be removed and an inward current of Ca^{2+} and Na^{+} accompanied by a outward current of K^{+}. Consequently, a depolarization of the postsynaptic membrane can occur. Subsequently, antagonists (MK-801 or PCP) can enter the ion channel and bind inside the ion channel. This blocks the receptor and stops the inward and outward currents of ion. Thus, no depolarization of the post synapse will occur. Modified after (Waxham, 1999)*

3.2 GABA

Gamma-Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the central nervous system and might play a part in schizophrenia and other neurological diseases. GABA, like glutamate, is synthesized from local precursors in the brain in collaboration between the neurons and astrocytes, the GABA-glutamine-glutamate cycle (Sonnewald et al., 1993; Waagepetersen et al., 2003).

3.2.1 The GABA-glutamine-glutamate cycle

GABA is derived from glutamate by the action of the neuronal enzyme glutamic acid decarboxylase (GAD) of which two isoforms exists, GAD_{65} and GAD_{67}. The different isoforms are expressed in different sub cellular localizations and have different regulatory properties (Martin and Rimvall, 1993). GAD_{65} is localized in the nerve endings and is connected to the vesicle membranes, while GAD_{67} is evenly distributed in the cytoplasm. The localization of GAD_{65} may indicate that this isoform may have a more prominent role than GAD_{67} in the synthesis of vesicular GABA. In this context it is interesting that GAD_{65} knockout mice develop seizures and the release of GABA is restricted (Tian et al., 1999). Studies have shown that a GAD_{67} knockout is lethal, thus the cytosolic pool of GABA is of great importance (Asada et al., 1997). During depolarisation of the GABAergic neuron, GABA is released into the synapse and binds to receptors on the postsynapse. The most important GABA receptor is the ionotropic $GABA_A$ receptor. Activation of this receptor will cause a flow of Cl^- into the cell and cause hyperpolarisation. $GABA_A$ receptors are common targets for a number of pharmacological agents, e.g benzodiazepines. Released GABA is rapidly removed from the synapse by reuptake into the presynaptic neuron or to a lesser extent by the astrocytes (Schousboe, 1981, 2003) via GABA transporters (GAT). Different subtypes of this transporter are found and are present on the GABAergic neurons, GAT-1, which is present on the GABAergic neuron, and to some extent on the astrocytes. Furthermore, released GABA taken up by the neurons can be stored and reused by the GABAergic neuron. In the astrocyte, GABA is metabolised by the enzymes Gamma-Aminobutyric acid transaminase (GABA-T) or succenic semialdehyde

dehydrogenase (SSADH) and converted to succinate. Succinate is an intermediate in the TCA cycle and after several steps it is converted to α-ketoglutarate, which is converted to glutamate, thereafter glutamine, this is known as the GABA shunt (see figure 1). Glutamine can then be transported back to the GABAergic neurone and by the enzyme PAG glutamine is metabolised to glutamate, and further on to GABA by the enzyme GAD. This is known as the GABA-glutamine-glutamate cycle (Waagepetersen et al., 2003) (See figure 2).

3.3 Monoamines

The monoamines consist of transmitters of similar structure; among them are dopamine and serotonin. These transmitters and transmitter systems have for years been in focus in several brain disorders such as Parkinson's disease, schizophrenia and depression. These neurotransmitter systems are in close connection with the GABAergic and the glutamatergic neurotransmitter systems (Schetz, 2007).

3.3.1 Dopamine (Schetz, 2007)

Dopamine is a cathcholamine that is distributed throughout the brain. Dopamine is known to play a part in several executive functions in the brain such as regulation of emotions, reward, cognition and motor control. Alterations of the dopaminergic system are known to be involved in the aetiology of several neurological diseases like Parkinson's and schizophrenia. Dopmaine is synthesized from L-DOPA via the enzyme aromatic amino acid decarboxylase (AAAD), and can further, as a step in removal and deactivation, be metabolised to 3,4-Dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA). Dopamine exerts its action trough binding and activation of specific receptors which is coupled to G proteins. The most common dopamine receptors are the D1 and D2 receptors. These receptors are coupled to different G proteins and have different effects when activated by dopamine. Activation of the D1 receptor mediates an excitatory effect, while activation of the D2 receptors mediates inhibitory control.

4. Animal models

Animal models serve as tools to recreate and mimic human diseases. However recreating human disease in its normal course in animals is difficult. Nevertheless animal models have shown to be useful and quite reliable. When choosing an experimental animal model for neurological and psychiatric disorders some criteria are important to follow (Lipska and Weinberger, 2000; Lipska, 2004).

1. The model chosen has to mimic the human disease targeted. In several neurological diseases, such as schizophrenia there are several cognitive alterations, which are difficult to mimic and monitor in an animal. Monitoring a distinct behaviour such as sexual pattern or fatigue can easily be performed, but the feeling of guilt, or hallucinations are difficult to monitor.

2. The model chosen has to involve as many inducing factors as possible that are thought to cause the modelled illness. The model should also reflect some aspects of the observed behaviour seen in the modelled disease. This are difficult to accomplish since knowledge about many of the underlying factors responsible for brain disorders are limited, nevertheless this is why animal models are useful.

3. The model chosen should be able to predict the therapeutic activity of drugs used to treat this disorder. This is an important point, because animal models lay the foundation for further clinical research and development of pharmacological treatments. Animal models responding to the same pharmacological treatment used in humans reflects a good model.

4.1 Schizophrenia

Schizophrenia is a life transforming illness that affects about 1% of the population. Those affected are highly dependent on medical care, and are often institutionalized (Bressan and Pilowsky, 2000). This illness is characterized by delusions, depression and cognitive impairment (positive and negative symptoms). The underlying cause of schizophrenia is not known, but several observations point towards genetic and neurodevelopment factors which may cause alterations in neurotransmitter homeostasis (Morrison and Murray, 2005). For decades the **dopamine hypotheses** have been the most established hypotheses for schizophrenia. This hypothesis proposes there is a hyperactivity of the dopamine transmission (Carlsson et al., 2001; Mozayani et al., 2003). However, antipsychotic drugs targeting dopamine receptors, mainly the D2 receptor (Laruelle et al., 2005; Schetz, 2007), are most effective at treating the positive but not the negative symptoms of schizophrenia (Tamminga and Carlsson, 2002). It has become apparent that glutamatergic neurons in the cortex and the hippocampus are modulated by dopamine (David et al., 2005), and input from glutamatergic neurons regulate the release of dopamine (Kegeles et al., 2000; Laruelle et al., 2003). Thus recently it has been hypothesized that alterations in the dopaminergic system might be downstream effects of altered glutamate metabolism (Laruelle et al., 2005). These transmitter systems are also in close interaction with the GABergic transmitter system. Authors have reported changes in GABAergic characteristics in schizophrenic patients (Gluck et al., 2002; Lewis et al., 2005; Lewis and Moghaddam, 2006) thus excitatory/inhibitory balances are disturbed. It is also assumed that disturbances of the GABAergic system may contribute to the sensory motorgating deficits and deficits in prepulse inhibition (PPI) observed in schizophrenia and several other psychiatric disorders (Heldt et al., 2004).

Several neurochemical hypotheses have been proposed to explain the origin of the symptoms seen in schizophrenia. One widespread hypothesis involves the glutamatergic systems, the **NMDA receptor hypofunction hypothesis**, originally proposed by Olney et al. (1995, 1999). The glutamatergic hypotheses of schizophrenia suggest that there is a dysfunction in the glutamatergic transmitter system involving the NMDA receptor.

These receptors are wide spread in the brain and play a major role in glutamatergic transmission, thus alterations of this receptor might have great impact on the transmitter homeostasis. Clinical studies have shown that exposure to NMDA receptor antagonist such as phencyclidine (PCP), ketamine or dizocilpine (MK-801) trigger symptoms resembling those seen in schizophrenia in healthy individuals (Javitt and Zukin, 1991; Bressan and Pilowsky, 2000) and exacerbate both positive and negative symptoms in patients with schizophrenia (Lahti et al., 1995; Olney et al., 1999; Rujescu et al., 2006).

4.1.2 Animal model of schizophrenia

For schizophrenia NMDA antagonists like PCP and MK-801 are widely used (Olney et al., 1999; Egerton et al., 2008). In rats these compounds cause behavioural changes like hyper locomotion, stereotyped behaviour, head weaving and ataxia. It is believed that these behavioural changes resemble psychosis experienced by schizophrenics. The MK-801 animal model of schizophrenia makes it possible to study the consequence of a down regulated NMDA receptor function on normal brain metabolism. Dopaminergic agonists and NMDA antagonists form the basis for the dopamine and glutamate models of schizophrenia. MK-801, is a non-competitive NMDA receptor antagonist and binds inside the ion channel of the receptor, and thus prevents the flow of calcium ions through the channel. It has been proposes that NMDA receptor hypofunction (the state induced in humans and rats when exposed to NMDA antagonists) is an important factor in this illness (Olney and Farber, 1995; Kehrer et al., 2008). Therefore it is believed that the disease can be modelled by blocking the NMDA receptor. NMDA receptor antagonists have been extensively studied for use in the treatment of diseases with excitotoxic components, such as stroke, and neurodegenerative diseases (Olney and Farber, 1995; Chen et al., 2008; van Marum, 2009). However NMDA antagonists like MK-801 have largely failed in clinical trials. Studies have shown that exposure to MK-801 induces schizophrenia-like symptoms in normal patients (Javitt and Zukin, 1991; Bressan and Pilowsky, 2000). These drugs also exacerbate both positive and negative symptoms in schizophrenics (Lahti et al., 1995; Rujescu et al., 2006). Thus, the NMDA receptor hypofunction hypothesis was developed (Olney et al., 1999).

In further search of the underlying causes of schizophrenia genetic animal models are useful. Here we used a genetic knock out of the GABA synthesizing enzyme GAD65. This animal model is not a model of schizophrenia *per se*, but it is a good model to elucidate the mechanisms of GABAergic synthesis and how the two different enzymes contribute to the synthesis and transmission of GABA. Several authors have reported deficits in the GABAergic system in patients with schizophrenia (Gluck et al., 2002; Lewis et al., 2005; Lewis and Moghaddam, 2006). Furthermore, deficits of both GAD isozymes have been reported in clinical studies (Todtenkopf and Benes, 1998; Akbarian and Huang, 2006). (For further description on GABA metabolism see chapter 4.2.1 The GABA-glutamate-glutamine cycle). Furthermore Heldt et al. (2004) reported PPI deficits in GAD65 knockout mice, interestingly these deficits were reversed using atypical antipsychotics such as clozapine. Deficiency in sensory motor gating mechanisms is seen in schizophrenia and number of other neuropsychiatric disorders (Braff et al., 2001). Despite these indications of disturbed GABA metabolism the link between alterations in GABAergic systems and schizophrenia is unclear. Thus this model is valuable to elucidate the mechanisms of GABAergic metabolism which might be a contributing factor to neuropsychiatric disorders.

5. Methods

5.1 ^{13}C labelled glucose and acetate as substrates for glial and neuronal metabolism

Acetate is selectively taken up by astrocytes by a specialized transport system, which is absent or less active in neurons, (Waniewski and Martin, 1998) whereas using ^{13}C NMRS it has been calculated that 65% of acetyl CoA derived from glucose is predominantly metabolized in the neuronal TCA cycle (Qu et al., 2000). Thus, by a simultaneous injection of [1-^{13}C]glucose and [1,2-^{13}C]acetate and NMRS analysis of brain extracts information about neuronal and astrocytic metabolism can be obtained in the same animal (Sonnewald et al., 1993; Waagepetersen et al., 2001b; Sonnewald et al., 2004). Injection of ^{13}C labelled glucose and acetate leads to efficient labelling of metabolites in the brain which can be seen in ^{13}C spectra of the brain extract. Label from [1-^{13}C]glucose can be quantified by analyzing the singlet peaks of the different compounds. The doublets are mostly derived from [1,2-^{13}C]acetate and thus astrocytic metabolism. As seen in figure 4, [1-^{13}C]glucose is converted to pyruvate *via* glycolysis and can form [3-^{13}C]alanine and [3-^{13}C]lactate. Pyruvate can enter the TCA cycle *via* [2-^{13}C]acetyl CoA. This will lead to the formation of [4-^{13}C]glutamate and glutamine or [2-^{13}C]GABA. Alternatively, pyruvate can be carboxylated by PC to oxaloacetate which will lead to the synthesis of [2-^{13}C]glutamate and glutamine or [4-^{13}C]GABA.

[1 – ^{13}C]glucose

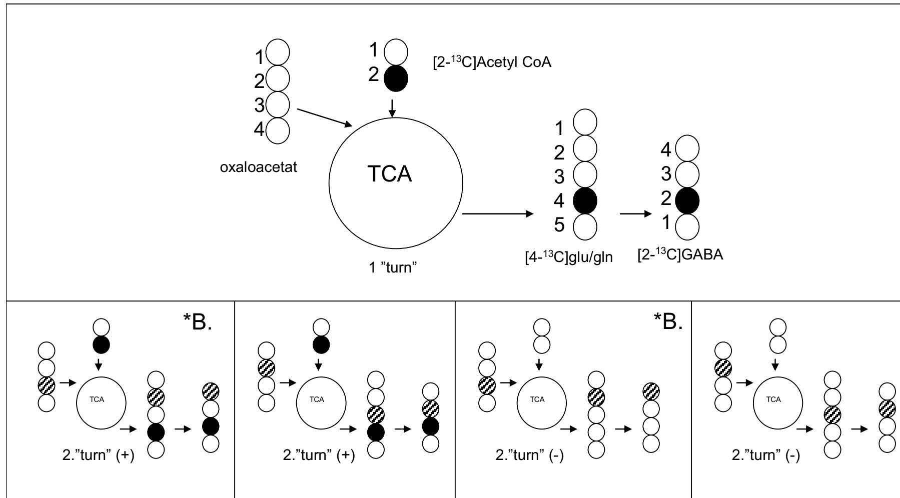


***Figure 4: Illustration of ^{13}C labelling originating from [1-^{13}C]glucose**. [1-^{13}C]Glucose via PDH provides labelling of the metabolites originating from the TCA cycle. The top part of the illustration shows labelling originating from the 1st turn of the TCA cycle. Black circles indicate ^{13}C. The lower part of the illustration shows labelling in metabolites from the 2nd turn in the TCA cycle (striped circles) with labelled (the two to the left) and unlabelled (the two to the right) acetyl CoA. In addition, sections marked ***B**, represent first turn via the PC pathway in astrocytes (striped circles). White circles indicate ^{12}C atoms. **Glu**;glutamate, **Gln**; glutamine, **PC**; pyruvate carboxylase, **PDH**; pyruvate dehydrogenase. **+/-** indicates whether oxaloacetate in the second turn will react with labelled or unlabelled acetyl CoA.*

As illustrated in figure 5 [1,2-^{13}C]acetate can be converted to acetyl CoA, although, the product, [1,2-^{13}C]acetyl CoA, will have two ^{13}C atoms, resulting in a doublet formation in the NMR spectra. Thus [4,5-^{13}C]glutamate and glutamine or [1,2-^{13}C]GABA are formed. Since both acetyl CoA and oxaloacetate can be labelled or unlabeled, the number of possible isotopomers of the metabolites derived from the TCA cycle is large. By comparing the doublets with singlets, detailed information about neuronal and astrocytic metabolism can be obtained. Thus [1,2-^{13}C]acetate

and [1-^{13}C]glucose will be used to analyze glial and neuronal metabolism in extracts from control rats and MK-801 administrated rats *in-vivo*.

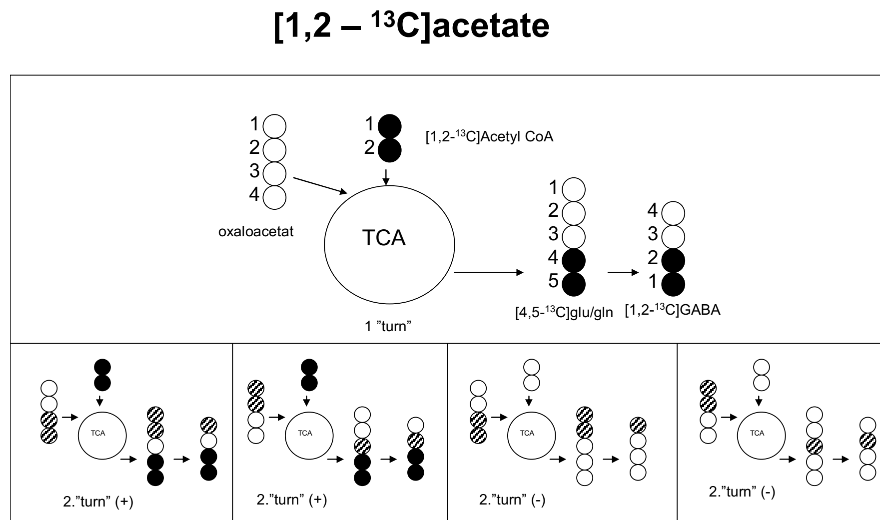


Figure 5: Illustration of ^{13}C labelling originating from [1,2-^{13}C]acetate. *Top part of the illustration represents ^{13}C labelling derived from the first turn of the TCA cycle (black circles). Bottom illustrations show labelling patterns after the second turn in the TCA cycle (striped circles). White circles indicate unlabeled ^{12}C atoms.* ***Glu****;glutamate,* ***Gln;*** *glutamine.* ***+/-*** *indicates whether oxaloacetate in the second turn will react with labelled or unlabelled acetyl CoA.*

5.2 Nuclear Magnetic Resonance spectroscopy

NMR spectroscopy has several appealing features for applications to metabolic studies. The nuclei that are most commonly used in NMR studies of biological tissues and extracts are ^{1}H, ^{31}P, and ^{13}C. ^{1}H and ^{31}P are isotopes with ~100% natural abundance, and therefore the most common nuclei to observe when examining metabolic interactions. However nuclei with low natural abundance such as ^{13}C, with a natural abundance of 1.1% are widely used in metabolic studies. ^{13}C NMR has a disadvantage

of low detection limit, thus it is of limited use for studies on endogenous metabolites unless they occur in large amounts (Bachelard and Badar-Goffer, 1993). This low natural abundance of ^{13}C can be turned to an advantage. ^{13}C-enriched precursors can be administrated for molecular pathway mapping. Therefore, ^{13}C NMR spectroscopy is a powerful tool for the analysis of brain metabolism and metabolic trafficking between different cellular compartments (Bachelard and Badar-Goffer, 1993).

5.2.1 Basic NMR principles (Bachelard and Badar-Goffer, 1993; Hornak, 1997-2008; Fribolin, 2004)

The nuclei of several isotopomers can from a classical point of view be described as small magnets spinning around their own axis. Nuclei with these properties are said to have a spin or a magnetic moment. The nuclei of ^{1}H and ^{13}C have a spin of ½; the spin can be positive or negative. Other nuclei such as ^{12}C have a spin of 0, and can thus not be detected by NMR. When a strong magnetic field (B_0) is applied to a sample containing these nuclei, the randomly orientated nuclei with a magnetic moment will absorb energy and align themselves along the axis of the B_0 field, either with or against its direction. The distribution of these directions have different energy states, some are aligned in the same direction as the applied field (α spin state) which is the lower energy state and some in the opposite direction (β spin state), which is the higher energy state. The nuclei will precess around the axis of B_0 with a certain frequency, the Larmor frequency. The rate of the frequency is proportional to the strength of the magnetic field and is expressed by equation; $\boldsymbol{\omega_0 = \gamma B_0/2\pi}$**.** Where ω_0 is the Larmor frequency in megahertz, B_0 is the magnetic field streng|th in tesla that the spin experiences, and γ the gyromagnetic ratio.

Applying a second magnetic field B_1 in form of a radiofrequency pulse (RF) perpendicular to the B_0 field, the spins will absorb energy and change their distribution towards the higher energy state. After the B_1 pulse have been turned off, the nuclei will return to their original state in a process called relaxation, and the excited spins are restored to their lower energy state. This shift in energy is the basis of a NMR signal; however this difference in energy is low, making the NMR an insensitive method.

During this energy release a current is generated in a detection coil as a signal, this signal is called the free induction decay (FID). The FID signal is stored in a computer where the signal is transformed by a Fourier transformation in order to generate a spectrum.

Different nuclei in the sample absorb different amounts of energy, depending on the electron density surrounding the given nuclei, thus giving a signal in the NMR spectra at different position depending on the density of the surrounding electrons. These different positions in the NMR spectra are called chemical shift and are reported in parts per million (ppm). This makes is possible to identify different metabolites and also distinguish between different nuclei in each metabolite. As seen in the spectra (figure 6) the appearances of the peaks differs, this is due to the magnetic properties of the nuclei and its neighbours. Molecules containing one ^{13}C with a ^{12}C atom as it neighbour it will appear as a singlet, if the neighbouring atom is a ^{13}C atom the signal will appear as a doublet or a triplet. This is due to a spin-spin-coupling which leads to splitting and displacement of the resonance from the central singlet resonance and a doublet will occur. ^{13}C- ^{13}C spin coupling make the detection of labels particularly specific because the likeliness of two naturally adjacent ^{13}C atoms in the same molecule are very small, which is why precursors like [1,2-^{13}C] acetate can be used as precursor in metabolic studies. The ^{13}C nuclei will also be affected by its neighbouring ^{1}H, which will lead to a splitting of the peaks. To avoid this splitting the spectra are proton decoupled. This is performed by exposure of radio waves at their Larmor frequency, so that the number of protons in the α and the β state is equal. Decoupling of the spectra will cause the peaks of some ^{13}C atoms to appear artificially large. This is called the nuclear overhouser effect (NOE). When analysing the spectra a factor for the NOE has to be applied, this factor vary between the different nuclei depending on conditions influencing the relaxation such as number of protons bound. The NOE and T_1 (T_1 is the longitudinal relaxation time) effects are accounted for by using correction factors based on acquired inversed gated spectra with no NOE effect and long relaxation delay (d1 = 30s) to ensure full relaxation of the ^{13}C nuclei of interest.

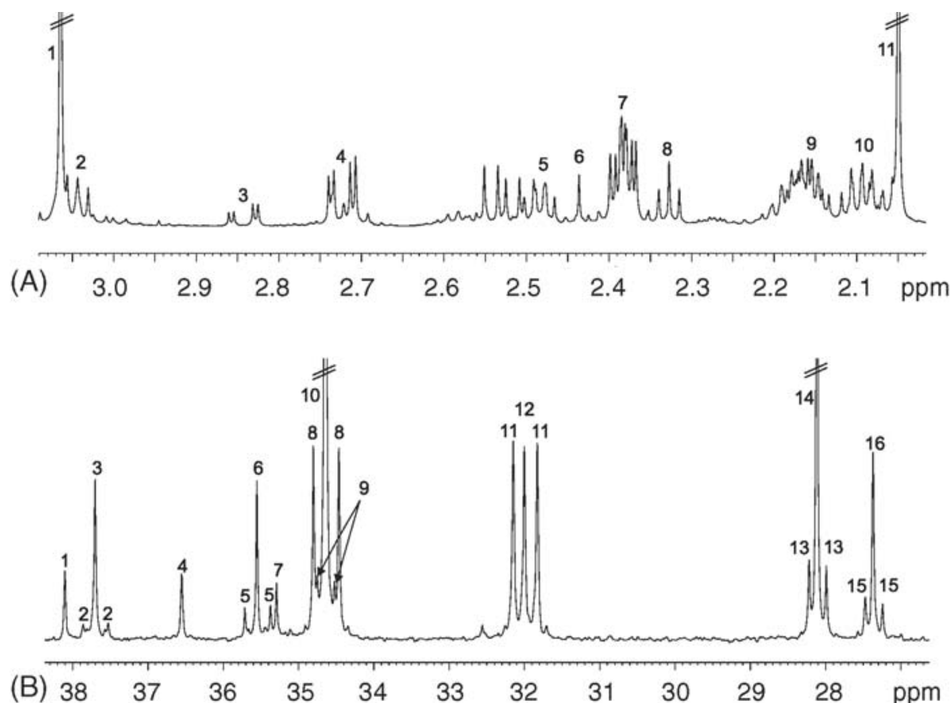


*Figure 6: **Typical ^{13}C NMR spectra of temporal lobe of animal injected with MK-801.** Animals were injected with [1-^{13}C]glucose and [1,2-^{13}C]acetate, 15 minutes later animals were euthanized by decapitation and their heads were snap frozen in liquid nitrogen. Temporal lobe were dissected and extracted with PCA. (A) ^{1}H NMR spectrum of temporal lobe extract from rat injected with MK801. Peak assignments; protons on 1, creatine C-3; 2, GABA C-3; 3, aspartate C-3; 4, NAA C-3; 5, glutamine C-4; 6, succinate C-2 or C-3; 7, glutamate C-4; 8, GABAC-2; 9, glutamine C-3; 10, glutamate C-3; 11, NAAC-6. (B) ^{13}C NMR spectrum of temporal lobe extract from animal injected with MK801. Peak assignments;1, creatine C-3; 2, aspartate C-3 (doublet); 3, aspartate C-3 (singlet); 4, taurine C-2; 5, GABAC-2 (doublet); 6, GABAC-2 (singlet); 7, succinate C-2 or C-3; 8,glutamate C-4 (doublet); 9, glutamate C-4 (doublet); 10, glutamate C-4 (singlet);11, glutamine C-4 (doublet); 12, glutamine C-4 (singlet); 13, glutamate C-3 (doublet); 14, glutamate C-3 (singlet); 15, glutamine C-3 (doublet); 16,glutamine C-3 (singlet). The singlets in the spectrum are mostly derived from [1-^{13}C]glucose and the doublets from [1,2-^{13}C]acetate (Eyjolfsson et al., 2006)*

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

Due to small variations in the experimental setup, see each experiment for details. However, samples were dissolved in D_2O (deuterated water) containing ethylene glycol as an internal standard. Proton decoupled ^{13}C NMR spectra were obtained using a BRUKER DRX- 600 or 500 spectrometer (BRUKER Analytik GmbH, Rheinstetten, Germany). Factors for the nuclear Overhauser and relaxation effects were applied to all

spectra. ^{1}H NMR spectra was obtained using the same spectrometer. Water suppression was achieved by applying a low-power presaturation pulse at the water frequency.

5.3 Gas chromatography- mass spectrometry (GC-MS) (McMaster, 1998)

The compounds of interest in this thesis were separated by gas chromatograph (GC) and converted into gas ions. These ions were sorted in the mass analyzer according to their mass-to-charge (m/z) ratios and then collected by a detector. Electrical signals were then recorded by the detection system, and the information was converted into a mass spectrum. Mass spectrometry (MS) is an analytical method based on the determination of atomic or molecular masses of individual compounds alone or in a mixture, and measures the mass-to-charge ratio of ions. This is achieved by ionizing the sample and separating ions of differing masses and recording their relative abundance by measuring intensities of ion flux. MS has a much higher sensitivity compared to many other methods such as magnetic resonance spectroscopy, thus it is used for analysis of metabolites with low concentration. However the disadvantage to NMR is that MS only gives the percent distribution of different masses (M which is the mass of the parent ion). Thus this method only tells you how many ^{13}C atoms are in the molecules. However this method does not give any information about the position of the ^{13}C atom in the molecule, and it does not differentiate between isotopomers containing the same number of ^{13}C in different positions. Therefore some information about what turn of the TCA cycle the ^{13}C labelled atom originates from is lost.

5.3.1 Experimental setup for Gas Chromatography - Mass Spectrometry (GC-MS)

Samples were analyzed using a GC (6890N, Agilent, USA) linked to a MS (5975B, Agilent, USA) with an electron ionization source. The percentual distribution of mass isotopomers for the derivatized amino acids was determined. See paper IV for further details.

5.4 High Performance Liquid Chromatography (HPLC)

HPLC (High Performance Liquid Chromatography) is an analytical method which in the present studies is used to quantify different amino acids and other metabolites in the tissue extracts. The metabolites are pre-column derivatized with *o*-phthaldialdehyde and subsequently separated on a ZORBAX SB-C18 (4.6×250mm, 5µm) column from Agilent using a phosphate buffer (50 mM, pH = 5.9), a solution of methanol (98.75 %) and tetrahydrofurane (1.25 %) as eluents. The separated metabolites are detected using a fluorescence detector and quantified by comparison to an external standard curve.

5.5 Statistics

The statistical difference between the groups was analyzed by students T-test. See different papers for further details. $P<0.05$ was considered significant.

6. Aims of studies

1. What are the glial-neuronal interactions in the MK-801 animal model of schizophrenia?

a) How are glial-neuronal interactions affected by a NMDA antagonist?

b) Is administration of MK-801 a good model for schizophrenia and what doses of repeated injections of MK-801 are best for modelling schizophrenia?

2. What are the glial –neuronal interactions in the GAD_{65} knockout mouse model?

a) How is the GABA metabolism affected by the absence of GAD_{65}?

3. Is there a common pattern of glial-neuronal interactions in these animal models?

7. Synopsis

Paper I

Repeated injection of MK-801: an animal model of schizophrenia?

Eyjolfsson E.M, Brenner E, Kondziella D, Sonnewald U.

Objective: The aim of this study was to investigate if administration of a low dose (0.1 mg/kg) of MK-801 produces symptoms resembling schizophrenia. We also wanted to elucidate how the neuronal-glial interactions were affected after repeated low dose administration.

Method: Rats were given i.p. injections of MK-801 (0.1 mg/kg) or saline on 6 consecutive days, the last dose together with [1-^{13}C]glucose and [1,2-^{13}C]acetate. Analyses of extracts from Frontal cortex plus cingulate and retrosplenial cortices (FCR) and temporal lobe (TL) were performed using ^{13}C and ^{1}H magnetic resonance spectroscopy.

Results: Altered behaviour was observed in some of the animals, and did not increase in severity after repeated injections. Metabolic changes were found in TL only. Increases in amounts and labelling of glutamate and glutamine from [1-^{13}C]glucose and [1,2-^{13}C]acetate were confined to TL.

Conclusions: Repeated injections of a low dose of MK-801 did not mimic the behavioural changes nor the metabolic changes seen in schizophrenia, but rather those seen after a single high dose (0.5 mg/kg) injection of MK-801 (Brenner et al., 2005). In this study we found that repeated injections of low doses (0.1 mg/kg) of MK-801 led to **hypermetabolism** and increased glutamatergic function. Thus, it appears that this model does not show the same pattern as seen in patients with schizophrenia but rather mimics the toxic effects of MK-801 possibly caused by increased glutamate release into the synaptic cleft.

Paper II

Glial-neuronal interactions are impaired in the schizophrenia model of repeated MK-801 exposure.

Kondziella D, Brenner E, Eyjolfsson E.M, Markinhuhta K.R, Carlsson M.L, Sonnewald U.

Objectives: The aims of this study were to investigate how neuronal-glial interactions were affected by administration of repeated high dose (0.5 mg/kg) of the NMDA antagonist, MK-801, and try to clarify if this model mimics schizophrenia.

Method: Rats were given i.p. injections of MK-801 (0.5 mg/kg) or saline on 6 consecutive days, the last dose together with [1-^{13}C]glucose and [1,2-^{13}C]acetate. Analyses of extracts from Frontal cortex plus cingulate and retrosplenial cortices (FCR) and temporal lobe (TL) were performed using ^{13}C magnetic resonance spectroscopy. HPLC was used to measure total amounts of amino acides and monoamines.

Results: Administration of MK-801 induced stereotypical behaviour like hyperlocomotion, ataxia, flat body posture and head waving. MK-801 affected metabolism in FCR to a larger extent than that in TL. In the FCR levels of glutamate were increased, whereas turnover of dopamine was unchanged. Glutamate and glutamine, derived from [1,2-^{13}C]acetate and thus astrocytes, were significantly decreased in FCR compared to controls. Labelling from [1-^{13}C]glucose and thus mostly neuronal metabolism was affected in the same brain region with decreased labelling of glutamate and GABA. Labelling and thus metabolism was unchanged in TL.

Conclusions: The present model of repeated injection of high doses of MK-801 resulted in increased amounts of glutamate. However, reduced ^{13}C labelling was observed in the same study, which might indicate a transition to reduced glutamate metabolism, and glutamate amounts seen in patients with chronic schizophrenia and consequently a **hypometabolism.** Main conclusion is that this model is a good model to mimic the first episode of schizophrenia or the transition phase before developing chronic schizophrenia.

Paper III

Altered Metabolism of Amino Acid Neurotransmitters and Dopamine in the Repeated MK-801 Animal Model of Schizophrenia

Eyjolfsson E.M, Nilsen L.H, Kondziella D, Brenner E, Sonnewald U, Håberg A.

Objective: The aim of this study was to further elucidate the metabolic changes seen after NMDA receptor hypofunction induced by MK-801 in more well defined brain areas than in paper II, and gain more accurate metabolic data.

Method: Repeated high-dose MK-801 exposure is an established model of schizophrenia and evokes positive, negative and cognitive symptoms. Rats received daily doses of MK-801 (0.5 mg/kg) i.p for 6 days. Frontal cortex (FCX), parietal and temporal cortex (PTCX), thalamus, striatum, nucleus accumbens (NAc) and hippocampus were examined using ^{13}C NMRS, high pressure liquid chromatography (HPLC) and Gas Chromatography and Mass Spectrometry (GC-MS).

Results: An overall decrease in glucose metabolism was demonstrated by reduced ^{13}C enrichment in lactate in all areas. Further MK-801 induced NMDA receptor hypofunction impaired transfer of lactate from astrocytes to neurons and decreased neurotransmitter turnover particularly in FCX. Mitochondrial metabolism was also impaired as shown by decreased labelling of TCA cycle intermediates in all brain areas. MK-801-treatement led to a significant decrease of glutamate, but not glutamine, turnover in FCX. This finding points to a specific reduction in the turnover of metabolic rather than the vesicular pool of glutamate. Further we found reduced ^{13}C labelling in GABA, lactate and NAA implying neuronal hypometabolism.

Conclusions: We have shown that perturbation of NMDA receptor function in the model of repeated injections of MK-801 caused changes not only in the glutamatergic and GABAergic system, but also in that of dopamine. Furthermore, it appears that glial transfer of lactate to neurons was impaired. Changes were most pronounced in FCX in analogy with results obtained in humans suffering from schizophrenia. The latter is certainly the case in the present study and most likely connected to the fact that NMDA receptor density is particularly high in the frontal lobe.

Paper IV

Knockout of GAD_{65} has major impact on synthesis from astrocyte-derived glutamine of vesicular GABA acting extrasynaptically

Walls A.B, Eyjolfsson E.M, Vestergaard H.T, Hansen S.L, Schousboe I, Schousboe A, Sonnewald U, Waagepetersen H.S

**Shared first authorship*

Objective: As GABAergic metabolism was shown to be altered in papers II and III as seen in patients with schizophrenia it was of interest to elucidate the involvement of GABA metabolism. Distribution of the GAD isoforms, the enzymes responsible for synthesis of GABA have been shown to be altered in patients with schizophrenia.
Method: A GAD_{65} knockout mouse model was used. Animals were injected with a combination of [1-^{13}C]glucose and [1,2-^{13}C]acetate 15 minutes prior to microwave fixation. Cortex was dissected and extracts were examined using ^{13}C NMRS and ^{1}H NMRS.
Results: Astrocytic metabolism seemed unaffected as percent enrichment of glutamate, glutamine and GABA derived from [1,2-^{13}C]acetate was unchanged in knockout animals. The percent enrichment with [3-^{13}C]lactate and [3-^{13}C]alanine labelled *via* glycolysis was dramatically decreased in GAD_{65} knockouts moreover TCA cycle activity was reduced indicating reduced glycolysis and **hypometabolism.** Furthermore, labelling derived from [1-^{13}C]glucose was reduced in glutamate, glutamine and GABA, pointing towards altered neuronal metabolism. The decrease in percent enrichment observed with [4-^{13}C]glutamine, which was comparable to the decrease observed in neuronal [4-^{13}C]glutamate, is most likely reflecting glutamine synthesis subsequent to glutamatergic neurotransmission and not astrocytic TCA cycle metabolism.
Conclusions: The absence of GAD_{65} led to a severe general neuronal **hypometabolism** as seen after repeated administration of high doses MK-801. Further we have provided evidence for a significant role of GAD_{65} in GABA homeostasis. It was revealed that GAD_{65} is crucial for maintenance of biosynthesis of synaptic GABA from astrocytic glutamine.

8. Discussion

Animal models serve as tools to recreate and mimic human diseases. However, recreating the “normal” course of human disease in animals is difficult. Still animal models have shown to be useful and quite reliable providing us with knowledge about brain pathology (Sonnewald and Kondziella, 2003; Melo et al., 2006; Kondziella et al., 2009). Several protocols exist for studies of glial-neuronal interactions in brain disorders. In the present thesis we used both pharmacological interventions mimicking one feature of schizophrenia, and a gene knock out model aimed at elucidating the role of GAD_{65}. Two different protocols for studying the effect of MK-801 were used to mimic schizophrenia. These models are useful to gain knowledge about how reduced NMDA receptor function affects the brain and its neurotransmitter homeostasis possibly leading to psychiatric disease. The interactions between neurons and astrocytes and how the synthesis of glutamate, glutamine and GABA are affected by MK-801 administration will be discussed. In papers I-III repeated injections of MK-801, simulating recurring psychotic episodes, were shown to alter glutamatergic and GABAergic function. Astrocytes are important in controlling glutamatergic and GABAergic homeostasis, thus it is not surprising that glial-neuronal interactions have been assigned a significant part in the pathophysiology of schizophrenia (Kondziella et al., 2007; De Keyser et al., 2008; Kolomeets and Uranova, 2009). In paper IV we used a genetic modified mouse model, GAD_{65} knockout mice, to gain knowledge abut GABA metabolism and elucidate the role of the two GAD isoforms, GAD_{65} and GAD_{67}. A deficit in the GABA metabolism has been reported in patients with schizophrenia (Gluck et al., 2002; Lewis et al., 2005; Lewis and Moghaddam, 2006). Furthermore, deficits of both GAD isoforms have been reported in clinical studies (Akbarian et al., 1995; Todtenkopf and Benes, 1998; Akbarian and Huang, 2006). GAD plays a central role in the synthesis of GABA and it is believed that the two isoforms have designated roles in this synthesis (Martin et al., 1991; Akbarian et al., 1995; Volk and Lewis, 2002). Clearly, a dysregulation of GAD would have profound consequences on metabolism contributing to the symptoms seen in schizophrenia (Dracheva et al., 2004).

Furthermore, GAD_{65} knockout mice display pronounced deficits in PPI (Heldt et al., 2004), a condition which has been shown in patients with schizophrenia (Braff et al., 2001; Geyer et al., 2001). Nevertheless PPI deficits have also been linked to several other neuropsychiatric disorders, implying a relationship between the metabolic and the synaptic events of the GABAergic system (Heldt et al., 2004). In paper IV we showed that disturbances of one of the GAD isozymes have great impact on the GABA homeostasis Patients with schizophrenia constitute a heterogeneous patient group with a large variety in symptoms and it is likely that the underlying causes of psychosis manifested in patients are induced by different mechanisms. Still, it is of great interest that blocking of the NMDA receptor, using repeated high dose of MK-801 causes hypometabolism as found in the GAD_{65} knockout model. Hypometabolism in FCX is a common finding in patients with schizophrenia (Andreasen et al., 1992; Schroeder et al., 1994).

Animal models have been particularly useful in the study of glial neuronal interactions as current in vivo methodology only to a limited degree allows for detailed studies of the interactions between these cell types in the brain.

8.1 Methodological considerations

To be able to interpret and compare the results obtained in papers I-IV, certain methodological considerations have to be taken into account.

Papers I and II differ from papers III and IV with regard to methodology used. In papers I and II all animals were decapitated and heads snap frozen in liquid nitrogen. From frozen brains, only larger brain areas can be dissected, consequently brain regions chosen in these studies contain parts of several other anatomically defined regions and also white matter. In papers III and IV, animals were euthanized using microwave fixation. This method instantly kills the animal and stops all enzyme activity and thus preserves the metabolites of interest. In addition, microwave fixation reduces the chance of post mortem metabolism in particular production of lactate (Caesar et al., 2008) and GABA. Due to the more firm consistency of the brain, this method allows a more

accurate dissection protocol. Therefore in paper III well defined small brain regions within anatomically defined borders were analysed with GC-MS and NMR, providing more accurate metabolic results for each brain area. Therefore employing the most appropriate method of termination is crucial in obtaining reliable results.

Paper III also differs from papers I, II and IV in that only [1-^{13}C]glucose was injected. Thus some information about astrocytic metabolism, function and neuronal utilisation of astrocytic metabolites was not obtained. In papers I, II and IV, [1,2-^{13}C]acetate in addition to [1-^{13}C]glucose was used. As described earlier, acetate is solely metabolised by astrocytes and thus provides information about metabolites derived from astrocytes.

Large amounts of data obtained in paper III (high dose MK-801, 0.5mg/kg) were derived using GC-MS. Some brain areas were studied using both NMR and GC-MS, but it was only possible to obtain NMR data from the larger brain areas such as FCX and PTCX. Consequently GC-MS, which is more sensitive, was used to analyse the smaller brain regions. Brain regions such as nucleus accumbens have a weight of ~10 mg, which is too small to be analysed by the NMRS instruments available to us, within a reasonable amount of time. Therefore GC-MS was used in paper III since it is sensitive and fast. However, GC-MS does not give any information about the position of the ^{13}C atom in the molecule, and it therefore does not differentiate between isotopomers containing the same number of ^{13}C but in different positions.

Results presented in paper IV are obtained from mouse brain, and are therefore not directly comparable to numbers in the previous studies (papers I-III). It is known from the literature that the distribution of the isozymes of GAD differs in different brain regions between species (Sheikh et al., 1999). However this study was performed to gain more knowledge about the general GABA metabolism and elucidate the particular role of GAD_{65} especially with regard to schizophrenia.

8.2 Effect of NMDA receptor blocking on cytosolic and mitochondrial metabolism

Large amounts of cortical glucose are predicted to be used to meet the demands of glutamatergic transmission (Attwell and Laughlin, 2001). Glucose metabolism is coupled to the glutamate–glutamine-GABA cycle, providing energy for uptake of extracellular glutamate and its conversion to glutamine in astrocytes (Dienel and Hertz, 2001). Astrocytic release of lactate is proposed to take place during brain activation and the lactate is subsequently taken up by neurons as an energy substrate (Dienel and Hertz, 2001).

In papers I and II, where we used decapitation and liquid nitrogen to gain brain tissue samples, no differences in alanine or lactate metabolism were found. Alanine and lactate are metabolites that can reflect glycolytic activety. It is not possible to measure pyruvate since the amount of it is low and it is chemically unstable. However, ^{13}C labelling in lactate is thought to reflect labelling in pyruvate (Schousboe et al., 1997). In both papers I and II, on the basis of unchanged ^{13}C enrichment of alanine and lactate we concluded that glycolysis was unaltered. However, the methods used to euthanize the animals can, as described in paragraphs above, lead to post mortem lactate production and cover up an actual decrease in lactate. In paper III, where a microwave fixation was used to euthanize the animals, we found decreased ^{13}C enrichment in lactate, pointing towards altered glycolysis. Similar findings were reported by Schroeder et al. (1994) in corresponding brain areas. Holmes et al. (2006) found a significant decrease in cerebrospinal fluid lactate levels in drug-naïve patients with first-episode schizophrenia. Based on the reports in the literature it seems that lactate levels fluctuate with disease duration (Holmes et al., 2006).

NAA, synthesized in mitochondria, has been suggested to reflect neuronal function and integrity (Moffett et al., 2007). Several studies have reported a reduced level of NAA in the temporal lobe and prefrontal cortex in patients with schizophrenia (Bertolino et al., 1996, 2000; Steen et al., 2005; Tanaka et al., 2006). Our results presented in paper III are in line with reports of decreased NAA levels in the prefrontal cortex of patients with schizophrenia during the early course of the illness (Cecil et al., 1999; Bustillo et al., 2002; Jessen et al., 2006). However in paper II, NAA was unchanged indicating a

normal metabolism, but there was reduced labelling of glutamate, GABA, and aspartate from [1-^{13}C]glucose (see paper I). This indicates that mitochondrial metabolism was affected. This unchanged level of NAA in paper II could be a result of a poor spatial resolution in the dissection protocol, thus a reduction of NAA in FCR in paper II could be hidden by unchanged NAA levels in surrounding defined brain regions. In paper III alterations in NAA, glutamate and GABA was all observed in FCX. In schizophrenia decreased levels of NAA seem to correlate with reduction of glutamate levels in frontal lobes (Ohrmann et al., 2005), impairment of cerebral blood flow (Bertolino et al., 2000), the extent of negative symptoms, and poor working memory (Moffett et al., 2007).

8.2 The effect of MK-801 on glutamate metabolism

Glutamatergic neurotransmission is compromised following administration of MK-801. We found that different dosage schemes of MK-801 had different effects on glutamate metabolism and glutamatergic function.

8.2.1 Hypermetabolism

The main findings in paper I were that repeated low dose (0.1 mg/kg) injections of MK-801 leads to hypermetabolism and thus an increased glutamate-glutamine cycling between neurons and astrocytes, as the labelling of both glutamine and glutamate was increased in temporal lobe (TE). An increase in glutamate labelling might over time lead to increased amounts of glutamate, which could have neurotoxic effects (Farber et al., 2002). Increased labelling from [1-^{13}C]glucose was also found in the same metabolites, whereas GABA labelling was unchanged. This will shift the balance between the excitatory and the inhibitory neurotransmitter possibly leading to overexcitability (Kehrer et al., 2008). These findings are similar to those reported by Brenner et al (2005) where a single high dose (0.5 mg/kg) of MK-801 also produced alterations restricted to the TE. Authors have reported morphological changes in form of reduced brain volume in *planum temporale*, such alteration may indicate neurotoxicity and consequently cell death (Yamasaki et al., 2007). It has been hypothesised that the neurotoxic effect of NMDA receptor antagonists is a result from

blockade of NMDA receptors on the GABAergic neurons. Such reduction of GABAergic inhibition on the downstream glutamatergic neurons indirectly leads to increased glutamate (Olney et al., 1999; Homayoun and Moghaddam, 2007). The results in paper I concur with this hypothesis and suggest that acute dose or repeated low dose of MK-801 is neurotoxic. It has been hypothesised that schizophrenia is a disorder of neurodegenerative processes. However, schizophrenic patients do not display the classical neurodegenerative features, but histophathological, neurochemical and neurostructural deficits are emerging (Jarskog, 2006). These findings are in line with our results of an increase in glutamate and thus the neurotoxic effects of NMDA blocking found after repeated low dose MK-801.

Table 1. *^{13}C NMRS and GC-MS results from experiments analyzing the glial- neuronal interactions using different protocols of MK-801 and a gene knockout model*

Isotopomers	Paper I		Paper II		Paper III		Paper IV
	Repeated low dose		**Repeated high dose**		**Repeated high dose**		**GAD65 knockout**
[1,2-^{13}C]acetate	FCR	TE	FCR	TE	FCX	PTCX	CX
[4,5-13C]glutamate		↑	↓				
[4,5-^{13}C]glutamine		↑	↓				
[1,2-^{13}C]GABA							
	Repeated low dose		**Repeated high dose**		**Repeated high dose**		**GAD65 knockout**
[1-^{13}C]glucose	FCR	TE	FCR	TE	FCX	PTCX	CX
[4-13C]glutamate		↑	↓		↓	↓	↓
[4-^{13}C]glutamine		↑					↓
[2-^{13}C]GABA			↓		↓	↓	↓
Lactate					↓	↓	↓
NAA					↓		

*Animals in paper I were treated i.p. with repeated low doses (0.1 mg/kg) MK-801 (Eyjolfsson et al., 2006) while animals in papers II and III were treated i.p with high doses(0.5mg/kg) (Kondziella et al., 2006 and Eyjolfsson et al 2009) every 24 h for 6 days. Animals in papers I and II were together with the last injection containing MK-801 or saline [1,2-^{13}C]acetate (504 mg/kg, 0.6 M solution) and [1-^{13}C]glucose (543 mg/kg, 0.3 M solution) Animals in paper III received only [1-^{13}C]glucose as labeled substrate after the last injection of MK-801 or saline. Animals in paper IV were GAD_{65} knockout animals, thus only receiving labeled substrates, [1,2-^{13}C]acetate (504 mg/kg, 0.6 M solution) and [1-^{13}C]glucose (543 mg/kg, 0.3 M solution). **FCR**; frontal/cingulate/retrosplenial cortices, **TE;** temporal lobe, **FCX;** frontal/motor cortex, **PTCX**; parietal and temporal cortex, **CX;** frontal/motor/parietal/temporal cortex.*

8.2.2 Hypometabolism

The main finding in paper II, was that repeated high doses of MK-801 resulted in a hypometabolism involving a decreased glutamate-glutamine cycle as observed by reduced labelling of glutamate and glutamine. Interestingly these alterations were confined to the FCX. Reports from several authors studying patients with schizophrenia report most pronounced alterations in frontal regions (Theberge et al., 2003; Ohrmann et al., 2005). Long term administration of MK-801 resulted in decreased ^{13}C labelling of glutamate in the frontal areas of the brain; this was also found by Moghaddam et al. (1997). In vivo ^{1}H NMRS study of drug naïve first episode schizophrenia patients, (Theberge et al., 2002) reported an increase of glutamine in cingulate cortex and thalamus.

Using high dose of MK-801 (paper III) we also found a hypometabolism in the FCX. In this study no differences were found in the total amounts of glutamate and glutamine. The observed changes in total amounts reported in papers I and II but not in paper III might be explained by differences in experimental setup with different dissection protocol and the use of microwave fixation in paper III (see further discussion in Methodological considerations). However in another study Theberge et al. (2003) reported decreased levels of glutamine and glutamate in the left anterior cingulate cortex of patients with chronic schizophrenia. In this study an average of ~15 years elapsed from the first psychotic episode occurred until amino acid levels were measured. In contrast in our experiments the study lasted for only six days, thus the results are not directly comparable. As highlighted, a repeated high dose MK-801 reflects the first episode phase of schizophrenia. In both studies using high dose MK-801, labelling derived from glucose was decreased in glutamate and GABA. Labelling in [4-^{13}C]glutamine was unchanged suggesting that transfer of glutamate from neurons to astrocytes was unaltered. In paper II we used [1,2-^{13}C]acetate as a metabolic tracer which provides us with valuable information about the metabolic activity in the astrocytes. Here we found a reduced labelling in [4,5-^{13}C]glutamate derived from [1,2-^{13}C]acetate, pointing towards either an impaired efflux of glutamine from astrocytes to neurons or an impaired conversion of glutamine to glutamate in neurons. Such

alterations suggest an altered glutamate-glutamine cycle after NMDA antagonist administration (Kondziella et al., 2006) and possibly schizophrenia. In paper III only [1-^{13}C]glucose was used and thus astrocytic metabolism was obscured by neuronal metabolism. Still the results in paper III support the finding of a hypometabolism. Nevertheless, paper III has the advantage of the usage of microwave fixations allowing a more precise dissection protocol, leading to more accurate results about the metabolites of interest.

8.3 The effect of MK-801 on GABA metabolism

GABAergic neurotransmission is also compromised following chronic administration of MK-801. Using the repeated high dose scheme (papers II and III) we showed that perturbation of NMDA receptor function causes changes in the GABAergic neurons. However, the changes in GABA metabolism were not seen in the repeated low dose model (paper I) or the acute model (Brenner et al., 2005). As discussed previously this model using low dose MK-801 does not mimic psychoses as seen in patients with schizophrenia. With recurrent administration of high doses of MK-801, changes in GABA metabolism became evident indicating that NMDA receptor hypofunction eventually leads to permanent GABAergic metabolic changes. Using high doses of MK-801 we found unchanged levels of GABA, however, [2-^{13}C]GABA derived from [1-^{13}C]glucose was reduced in the frontal regions (FCR and FCX). GABA activity might be particularly important in the frontal regions and in the development of the behavioural and cognitive symptoms seen in patients with schizophrenia. This is supported by findings by Theberge et al. (2003) and Ohrman et al. (2005), who found most pronounced alterations in FCR of patients with schizophrenia. The frontal regions have a high density of NMDA receptors which exhibit particular sensitivity to MK-801 administration (Coyle, 2004). NMDA receptor inhibition was shown to decrease GABAergic turnover and thus, possibly GABAergic transmission. Del Arco and Mora, (2002) showed that glutamate, through the activation of both NMDA and AMPA/kainate ionotropic receptors, facilitated GABAergic transmission in the prefrontal cortex. Deficits of GABAergic neurotransmission (Gluck et al., 2002; Lewis et al., 2005; Lewis and Moghaddam, 2006), and of both GAD isoforms have been

reported in clinical studies (Akbarian et al., 1995; Todtenkopf and Benes, 1998). (*See chaper* 3.2 GABA for further details). A dysregulation of any of the GAD isoforms could influence brain metabolism contributing to the symptoms seen in schizophrenia (Dracheva et al., 2004). As discussed in paper IV knockout of the GAD isozymes has great impact on GABA homeostasis. Interestingly studies of the GAD_{65} knockout mice show signs of hypometabolism involving glycolysis as well as TCA cycle activity; this was also seen after repeated administration of high dose MK-801.

8.4 The effect of MK-801 on Dopamine metabolism

Dopamine and hyperdopaminergia has been in the main target for treatment of schizophrenia for several years. It has become apparent that dopamine is an important modulator of the glutamatergic neurons in the cortex and the hippocampus (David et al., 2005), and also input from glutamatergic neurons regulate the release of dopamine (Olney et al., 1999; Kegeles et al., 2000). Thus recently it has been hypothesized that alterations in the dopaminergic system might be downstream effects of altered glutamate metabolism (Laruelle et al., 2005).

In papers II and III we measured concentration of dopamine. In paper II no differences were found whereas an increase was observed in paper III. This inconsistency may be related to the difference in experimental protocols used in the two papers. In the latter study we found that a hypometabolism of glutamate, lead to a hyperdopaminergia in FCX, as there was observed an increase in dopamine and its degradation product DOPAC levels, this is in line with Loscher et al. (1991). This increased release of dopamine in FCX, as a consequence of hypometabolism in FCX, could be explained by polysynaptic networks in the brain. It is known that glutamatergic neurons have a modulatory effect on the dopaminergic system (Olney et al., 1999; Kegeles et al., 2000). Using repeated high dose of MK-801 we directly affect the glutamatergic system, thus the observed alterations in dopamine is necessarily downstream of glutamatergic deficiency. This could also explain why current antipsychotics, targeting the dopaminergic system are most effective treating the positive but not the negative symptoms seen in schizophrenia (Tamminga and Carlsson, 2002). One could thus

assume that medication treating the glutamatergic or the GABAergic system is of great therapeutically value.

8.5 What is the preferred model to mimic schizophrenia?

The ability of MK-801 to effectively model the aspects of schizophrenia varies according to dose and injection scheme. Several different protocols exists, and there are indications that these protocols affect the glutamate and GABA system differently (Brenner et al., 2005; Eyjolfsson et al., 2006; Kondziella et al., 2006). Acute injection is a sudden alteration of the transmitter system, but schizophrenia develops over longer time periods. Therefore repeated administration of the same dose might alter the transmitter system in a way that it is behaving as a disturbed network would do in patients with schizophrenia. Metabolic alterations of other brain regions including the thalamus and the striatum have been observed (Javitt, 2006; Beasley et al., 2009) further suggesting alterations in polysynaptic networks between brain regions rather than in one single brain area (Homayoun and Moghaddam, 2007).

It has been speculated that an acute dose might mimic an acute psychotic episode (Manahan-Vaughan et al., 2008). Based on the similarities in the results after repeated low dose in paper I and acute high dose (Brenner et al., 2005), one could speculate that repeated low doses of MK-801 rather than or in addition to being a toxic model, represents the initiating phase of psychosis. Therefore this model may represent the changes occurring in the brain before patients is diagnosed as schizophrenic. Gao et al. (1993) found that acute high dose of PCP, a NMDA antagonist resembling MK-801, induced hypermetabolism after acute dosage, coinciding with out finding after repeated low dose of MK-801. Schizophrenia is a disease that develops over a long time span and possibly the metabolic alterations leading to the disorder initiated before birth (Jarskog, 2006). Therefore chronic administration of MK-801 appears more suitable to mimic natural development of schizophrenia. To be diagnosed as schizophrenic, recurrent psychoses are required. Consequently one could hypothesize that if treatment of these animals with low dose of MK-801 was continued over a longer period of time,

these animals might show the same metabolic alterations found using higher doses of MK-801 as discussed in papers II and III.

After repeated high dose MK-801 we found a hypometabolism, the core element of schizophrenic pathology (Weinberger et al., 1986; Williamson, 1987). As discussed in earlier paragraphs, repeated high dose administration of MK-801 produced some of the neurochemical and behavioural alterations found in schizophrenia. Although our results indicate that a repeated high dose MK-801 reflects the first episode phase of schizophrenia rather than the chronic phase of schizophrenia. The administration scheme used in our studies with a repeated high dose could thus be a subchronic model. Consequently one could assume that a prolonged administration of MK-801 e.g. 3 weeks would better reflect the chronic phase of schizophrenia. Studies of such recurrent psychosis are not limited by ethical considerations. Between injections animals treated with MK-801 eat, drink, groom and behave in the same manner as their untreated littermates. Although time consuming it might be worthwhile to consider using such model something to elucidate the mechanisms behind schizophrenia and psychosis. Some authors have proposed a developmental hypothesis of schizophrenia (Jarskog, 2006) where a disruption of the NMDA receptor already at the post natal stage is postulated. These two hypotheses are easily compatible, as one could start a low administration of MK-801 already at postnatal stage and continue this over a longer time span. A chronic model is vital if one want to alleviate the symptoms seen in schizophrenia, since patients with schizophrenia are not attended to before schizophrenia is established.

8.6 Regional effects of different doses of MK-801

Different doses of MK-801 might have specific regional effects due the diversity of neuronal pathways and/or the sensitivity of the NMDA receptor to MK-801 on neurons in different brain regions. It has been shown that populations of NMDA receptors in the different brain regions have dissimilar properties to be inhibited by MK-801. The NMDA receptors expressed on GABAergic neurons in the frontal regions are particularly vulnerable to MK-801 (Coyle, 2004). The different dose schemes may thus affect different neuronal pathways. Blocking the NMDA receptor on inhibitory GABAergic interneurons could indirectly increase the glutamate release of the downstream glutamatergic neuron, leading to neurotoxicity (Farber et al., 1995; Olney and Farber, 1995; Homayoun and Moghaddam, 2007). In contrast, blocking the NMDA receptor at the second glutamatergic neuron at the end of this chain might have a neuroprotective effect. In the studies by Brenner et al. (2005) and our study using repeated low dose MK-801 (paper I), metabolic changes were found in TL only. Moreover repeated high doses had lesser effect on the temporal areas, but led to significant alterations in the frontal regions (CRSC and FCX). In this context it is interesting that MK-801 directly injected into the FCX does not induce the same neurotoxic effects found with similar repeated doses (Farber et al., 2002). Thus it could be hypothesized in order to obtain a neurotoxic effect, a blockade of the NMDA receptor outside the FCX is required, indicating polysynaptic network disturbances. Different doses of MK-801 may cause similar reactions within FCX, but may affect other areas in the brain differently. Repeated high dose of MK-801 will most likely affect the polysynaptic connections in a manner affecting mostly FCX, low dose of MK-801 may instead affect other polysynaptic networks leading to alterations in the TE. These findings may imply that specific polysynaptic networks have to be “kindled” to provoke the symptoms seen in schizophrenia.

9. Conclusions

- Repeated low dose (0.1 mg/kg) MK-801 administration led to **hypermetabolism** and increased glutamate glutamine cycle activity, thus it was concluded that this model represent the toxic effects of MK-801.

- Repeated high dose (0.5 mg/kg) MK-801 administration led to glucose **hypometabolism**. We concluded that this is the most accurate way to mimic psychoses seen in first episode patients or those in the transition phase to schizophrenia.

- Polysynaptic networks seemed affected after repeated MK-801 administration, as several neurotransmitter systems, including the GABAergic, glutamatergic and the dopaminergic system, sere disturbed

- Repeated high doses (0.5 mg/kg) of MK-801 led to altered cytosolic and mitochondrial metabolism, and reduced lactate transfer from astrocytes to neurones.

- We found a neuronal **hypometabolism** in the GAD_{65} knockout mice, as seen after repeated administration of high dose (0.5 mg/kg) MK-801.

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Repeated injection of MK801: An animal model of schizophrenia?

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Abstract

Glutamate-induced neurotoxicity plays an important role in neurological and psychiatric diseases. Thus, much attention has been given to the potential neuroprotective role of glutamate receptor antagonists, especially to those acting on the *N*-methyl-D-aspartate (NMDA) subtype. However, in addition to their neuroprotective potential, these compounds have also neurotoxic and psychotogenic properties. In the present study we used repeated injections of MK801 to examine if this non-competitive NMDA receptor antagonist could be used to produce schizophrenia-like alterations in behavior and brain metabolism in animals. Rats were given injections of MK801 (0.1 mg/kg) on six consecutive days, the last dose together with [1-^{13}C]glucose and [1,2-^{13}C]acetate, to probe neuronal and astrocytic metabolism, respectively. Analyses of extracts from parts of the frontal cortex plus cingulate and retrosplenial cortices and temporal lobes were performed using ^{13}C and ^{1}H magnetic resonance spectroscopy. Changes in glutamate and glutamine were restricted to the temporal lobe, in which amounts and labeling from [1-^{13}C]glucose and [1,2-^{13}C]acetate were increased compared to control. Locomotor activity was slightly higher in rats treated with MK801 compared to untreated animals. Metabolic changes did not resemble the alterations occurring in schizophrenia and those after repeated high dose (0.5 mg/kg) [Kondziella, D., Brenner, E., Eyjolfsson, E.M., Markinhuhta, K.R., Carlsson, M., Sonnewald, U., 2005. Glial–neuronal interactions are impaired in the schizophrenia model of repeated MK801 exposure. Neuropsychopharmacology, Epub ahead of print] but rather those caused by MK801 seen after a single high dose (0.5 mg/kg) [Brenner, E., Kondziella, D., Haberg, A., Sonnewald, U., 2005. Impaired glutamine metabolism in NMDA receptor hypofunction induced by MK801. J. Neurochem. 94, 1594–1603.].
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1. Introduction

In order to establish new treatments, *N*-methyl-D-aspartate (NMDA) and other glutamate receptor antagonists have been tested clinically in many CNS disorders (Bensimon et al., 1994; Bullock et al., 1999; Davis et al., 2000). The principal therapeutic strategy is to reduce the neurotoxicity of excessive glutamate release. Some authors reported on the neuroprotective potential of glutamate antagonists (for review see Himmelseher and Durieux, 2005) some on lack of effect (Muir and Lees, 2003) and some revealed additional neurotoxic potential of glutamate/NMDA antagonism (Olney and Farber, 1994; Farber et al., 1996, 1998, 2002; Kim et al., 1999). Results of clinical studies using NMDA antagonism have been quite disappointing, although with increasing knowledge about the complexity of NMDA receptor subtypes this might change in the future. For a review on "the enormous potential of NMDA receptor antagonists" see Smith (2003).

NMDA antagonists have found use as tools to produce schizophrenia-like symptoms in animals. Several transmitter systems have been implicated in the pathogenesis of schizophrenia and combined dysfunction of the glutamate and dopamine systems has been suggested (Javitt and Zukin, 1991; Olney and Farber, 1995; Carlsson and Carlsson, 1999; Flores and Coyle, 2003). Disturbed glutamatergic neurotransmission is especially relevant in patients with significant negative symptoms and cognitive impairment (Goff and Coyle, 2001; Tsai and Coyle, 2002; Harrison and Weinberger, 2005). Furthermore, lower glutamate levels in the cerebrospinal fluid of schizophrenia patients have been reported as well as changes in the metabotropic and the ionotropic glutamate receptors in postmortem brain tissue (reviewed by Tamminga, 1998).

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In addition to the prefrontal cortex, the bulk of the data concerns the hippocampus (Heckers and Konradi, 2002; Harrison et al., 2003). Interestingly, all the known susceptibility genes for schizophrenia act on glutamatergic synaptic transmission (Harrison and Weinberger, 2005). However, reduced GABA synthesis has also been reported in schizophrenic patients (Lewis et al., 2005).

Animal models can help to better understand and find ways to cure human diseases. We have investigated the use of MK801 as a model of schizophrenia using different protocols (Brenner et al., 2005; Kondziella et al., 2005). Neurometabolite concentrations and turnover were assessed by high field ^{13}C and ^{1}H NMR spectroscopy and analyzed with special emphasize on glial–neuronal interactions. Due to the close connection of astrocyte metabolism and the synthesis of glutamate and GABA in neurons, there are good reasons to hypothesize an astrocytic role in the pathophysiology of schizophrenia and glutamate dysfunction (Kondziella et al., 2005). Earlier we have injected a single, relatively high dose of MK801 (0.5 mg/kg) and observed distinct behavioral abnormalities and increased levels of glutamate, glutamine and GABA combined with increased labeling of these amino acids mostly in temporal lobe (Brenner et al., 2005). After repeated injections of the same dose, increased levels of glutamate were found in the frontal cortex plus cingulate and retrosplenial cortices (FCR) but not in temporal lobe (Kondziella et al., 2005). To determine whether also a lower dose would produce schizophrenia-like alterations, rats were injected with 0.1 mg/kg MK801 every day for six consecutive days. Metabolite composition was analyzed by ^{13}C and ^{1}H NMR spectroscopy.

2. Materials and methods

2.1. Materials

Male Sprague Dawley rats (with an average weight of 250 g) were obtained from Möllegaard Breeding centre, Copenhagen, Denmark. Animals were housed in individual cages at a constant temperature of 22 °C with a 12 h light/dark cycle and a humidity of 60%. Animals had free access to food and water. [1-^{13}C]glucose, [1,2-^{13}C]acetate and D_2O (99.9%) were purchased from Cambridge Isotopes Laboratories (Woburn, MA, USA); ethylene glycol from Merck (Darmstad, Germany); MK801 (Diozocilpine; [5*R*,10*S*]-[+]-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclopheten-5,10-imine), from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of the purest grade available from local commercial sources.

2.2. Dosing and experimental design

The experimental design was approved by the Norwegian Animal Research Authorities Welfare and the local ethics committee. Animals were injected with saline or MK801 (0.1 mg/kg body weight) intraperitoneally every day for six days. The last dose of saline or MK801 was given together with [1-^{13}C]glucose (543 mg/kg, 0.3 M solution) and [1,2-^{13}C]acetate (504 mg/kg, 0.6 M solution). Twenty minutes later animals were decapitated, heads were snap frozen in liquid nitrogen and stored at −80 °C. Brains were removed, and two areas of each hemisphere were dissected. The first area included the cingulate, the retrosplenial and parts of the frontal cortices (FCR). The second area, the temporal lobe, was dissected by a horizontal cut from the most lateral point of the hemisphere extending approximately 3 mm medially and a second sagittal cut extending ventrally through the whole brain. The resulting sample consisted of the temporal cortex, piriform cortex, entorhinal cortex, amygdala and parts of the hippocampus. The dissection was performed on ice while the brains were still frozen. Thereafter, brain tissue was homogenized in 7% perchloric acid and centrifuged at $4000 \times g$ for 5 min. The procedure was repeated, the supernatants pooled and neutralized with 1 M KOH followed by centrifugation and lyophilization.

2.3. Evaluation of behavior

Cages were divided into four equally large areas by imaginary lines. After each drug administration locomotor activity was scored by counting how often the animals crossed the lines during a specific time interval.

2.4. ^{13}C NMR spectroscopy and ^{1}H NMR spectroscopy

Samples were dissolved in 200 μl D_2O (deuterated water) containing ethylene glycol 0.1% as an internal standard.

Proton decoupled ^{13}C NMR spectra were obtained using a BRUKER DRX-600 spectrometer (BRUKER Analytik GmbH, Rheinstetten, Germany). Scans were accumulated with a 30° pulse angle and 30 kHz spectral width with 64 K data points. The number of scans was 10,000. The acquisition time was 1.08 s, the relaxation delay 0.5 s. Factors for the nuclear Overhauser and relaxation effects were applied to all spectra.

^{1}H NMR spectra was obtained using the same spectrometer. Scans were accumulated with a pulse angle of 90° and a spectral width with 32 K data points. The number of scans was 400. Acquisition time was 1.36 s and relaxation delay was 10 s. Water suppression was achieved by applying a low-power presaturation pulse at the water frequency.

2.5. Data analysis

The amounts of ^{13}C and ^{1}H in the different metabolites were quantified from integrals of the relevant peaks obtained from NMR spectra with ethylene glycol as an internal standard. Some spectras were not included in the analysis due to methodological error. All results are given as mean ± standard deviation. Statistics were performed using Student's *t*-test, $p < 0.05$ was regarded as significant.

3. Results

Repeated low dose injections of MK801 altered the behavior of some animals. Locomotor activity was slightly increased in 3–4 animals at any given time point compared to control (results not shown). Successive injections did not lead to increased activity of the individual animal.

A typical ^{1}H NMR spectrum is depicted in Fig. 1A, whereas Fig. 1B shows a ^{13}C NMR spectrum from an extract of temporal lobe from a MK801 injected rat. ^{13}C labeled amino acids and other small molecules derived from [1-^{13}C]glucose or [1,2-^{13}C]acetate are clearly detected. In order to interpret the ^{13}C NMR spectra it is necessary to analyze the metabolic pathways for [1-^{13}C]glucose (Fig. 2A) and [1,2-^{13}C]acetate (Fig. 2B).

When [1-^{13}C]glucose and [1,2-^{13}C]acetate are administered simultaneously, metabolic interactions between astrocytes and neurons can be studied in the same animal. This is due to the fact that acetate is exclusively taken up by astrocytes, while the major part of acetyl-CoA derived from glucose is used in neurons. Label from [1-^{13}C]glucose and thus neuronal metabolism can be quantified by analyzing the singlet peaks in the spectrum. In contrast, the doublets seen in the spectra are mainly derived from [1,2-^{13}C]acetate and thus astrocytic metabolism. In neurons [1-^{13}C]glucose is converted to [3-^{13}C]pyruvate which can be transformed to [3-^{13}C]lactate

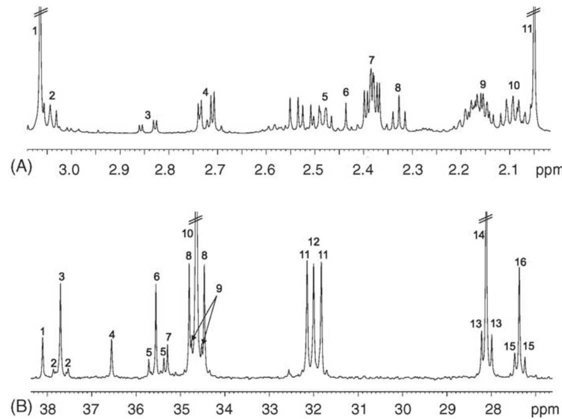

Fig. 1. (A) ^{1}H NMR spectrum of temporal lobe extract from rat injected with MK801. Peak assignments; protons on 1, creatine C-3; 2, GABA C-3; 3, aspartate C-3; 4, NAA C-3; 5, glutamine C-4; 6, succinate C-2 or C-3; 7, glutamate C-4; 8, GABA C-2; 9, glutamine C-3; 10, glutamate C-3; 11, NAA C-6. (B) ^{13}C NMR spectrum of temporal lobe extract from rat injected with MK801 together with [1-^{13}C]glucose and [1,2-^{13}C]acetate. Peak assignments; 1, creatine C-3; 2, aspartate C-3 (doublet); 3, aspartate C-3 (singlet); 4, taurine C-2; 5, GABA C-2 (doublet); 6, GABA C-2 (singlet); 7, succinate C-2 or C-3; 8, glutamate C-4 (doublet); 9, glutamate C-4 (doublet); 10, glutamate C-4 (singlet); 11, glutamine C-4 (doublet); 12, glutamine C-4 (singlet); 13, glutamate C-3 (doublet); 14, glutamate C-3 (singlet); 15, glutamine C-3 (doublet); 16, glutamine C-3 (singlet). The singlets in the spectrum are mostly derived from [1-^{13}C]glucose and the doublets from [1,2-^{13}C]acetate.

or [3-^{13}C]alanine or enter the TCA cycle as labeled [2-^{13}C]acetyl-CoA. The latter will lead to formation of [4-^{13}C]glutamate, [4-^{13}C]glutamine and [2-^{13}C]GABA. In addition, in astrocytes [1-^{13}C]glucose can be converted to [3-^{13}C]oxaloacetate by pyruvate carboxylase (PC). This will lead to labeling of [2-^{13}C]glutamate and [2-^{13}C]glutamine, and [4-^{13}C]GABA. [1,2-^{13}C]acetate can be converted to [1,2-^{13}C]acetyl-CoA. Labeled acetyl-CoA enters the TCA cycle and can lead to formation of [4,5-^{13}C]glutamate, [4,5-^{13}C]glutamine or [1,2-^{13}C]GABA.

Injection of MK801 lead to metabolic changes in temporal lobe only. Thus, no results from FCR are shown. In temporal lobe there was an increase in the labeling of glutamate and glutamine from both [1-^{13}C]glucose and [1,2-^{13}C]acetate, compared to controls (Fig. 3A and B). Aspartate labeling from [1-^{13}C]glucose was increased in the MK801 treated rats, whereas GABA labeling was unaltered.

As can be seen in Table 1, MK801 administration resulted in an increase in the amounts of glutamate, glutamine and succinate in temporal lobe. The amounts of GABA and aspartate were unaffected (Table 1).

Table 1
Amounts of metabolites (μmol/g) in extracts from the temporal lobe of controls and rats treated with MK801

	Control[a]	MK801
Glutamate	10.0 ± 1.2	$11.5 \pm 1.4^{*}$
Glutamine	3.6 ± 0.5	$4.3 \pm 0.6^{**}$
GABA	2.0 ± 0.4	2.3 ± 0.6
Aspartate	2.6 ± 0.4	2.9 ± 0.5
Succinate	0.7 ± 0.2	$0.9 \pm 0.2^{*}$

Animals were injected with saline ($n = 8$) or 0.1 mg/kg MK801 ($n = 10$) intraperitoneally every day for six days. Amounts of metabolites were analyzed with ^{1}H NMR spectroscopy. Metabolites were quantified using the protons on; glutamate C-4; glutamine C-4; GABA C-2; aspartate C-3; succinate C-2/3 (for details see Section 2). The results are expressed as mean ± S.D. and were analyzed with the Student's *t*-test.

[a] Values from Kondziella et al. (2005).

* $p < 0.05$ significant difference between control and MK801.

** $p < 0.02$ significant difference between control and MK801.

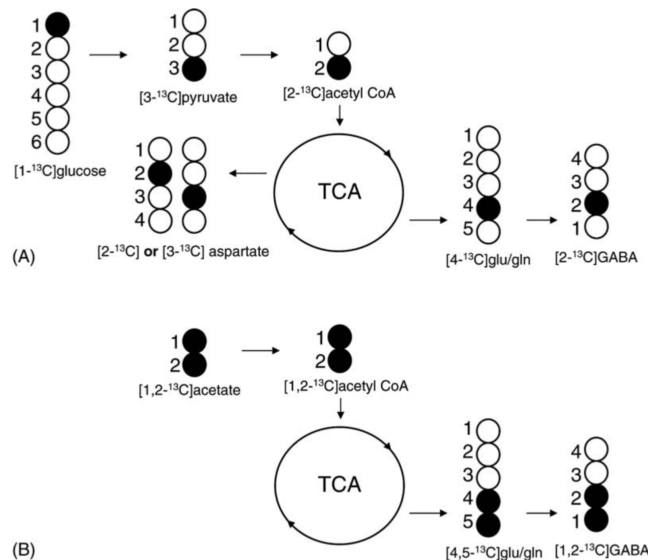

Fig. 2. Schematic presentation of isotopomers of glutamate, glutamine, GABA and aspartate derived from [1-^{13}C]glucose (A) and [1,2-^{13}C]acetate (B).

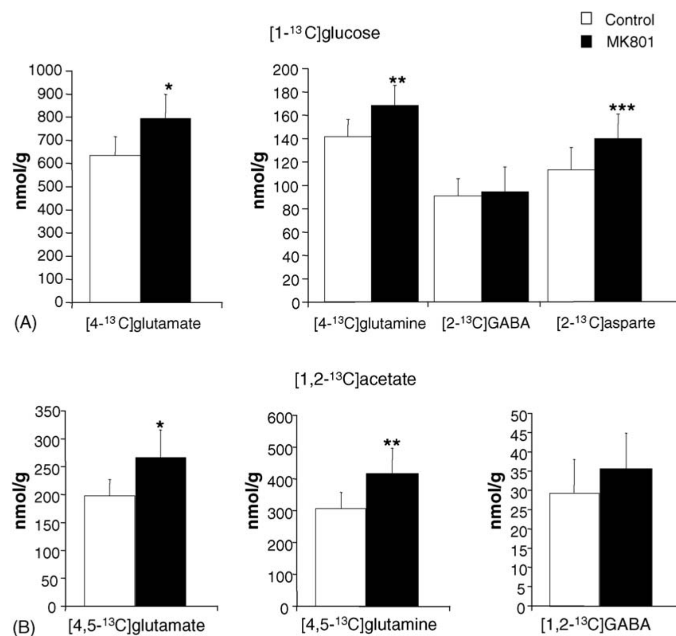

Fig. 3. Amounts (nmol/g brain tissue) of metabolites derived from (A) [1-^{13}C]glucose and (B) [1,2-^{13}C]acetate in extracts from the temporal lobe of rats treated with MK801 and controls. Animals were injected with saline ($n = 5$) or 0.1 mg/kg MK801 ($n = 7$) intraperitoneally every day for six days, the last day animals received [1-^{13}C]glucose and [1,2-^{13}C]acetate. Analyses were performed using ^{13}C NMR spectroscopy (for details see Section 2). The amount of ^{13}C in a metabolite was not corrected for natural abundance. The results are expressed as mean ± S.D. and were analyzed with the Student's t-test, control values from Kondziella et al. (2005). $^{*}p < 0.02$, $^{**}p < 0.03$, $^{***}p < 0.05$ significant difference between controls and MK801.

4. Discussion

NMR spectroscopy analyses of neurometabolites in brain from MK801 injected rats, a model of NMDA/glutamate-hypofunction and schizophrenia have provided valuable information about disturbances in amino acid neurotransmitter metabolism. Given once at 0.5 mg/kg, MK801 produced mainly changes in the temporal lobe with increased glutamate and glutamine concentrations and labeling from [1-^{13}C]glucose (Brenner et al., 2005). Similar changes were detected in the present study, when MK801 was injected repeatedly at 0.1 mg/kg every day for six days and changes were restricted to the temporal lobe. However, using 0.5 mg/kg repeatedly (Kondziella et al., 2005) instead of 0.1 mg/kg, resulted in changes in the FCR, thereby mimicking alterations observed in patients with schizophrenia (Bartha et al., 1997; Theberge et al., 2002; Tebartz van et al., 2005).

4.1. Glutamate and glutamine concentrations

Repeated injection of 0.1 mg/kg MK801 increased glutamate and glutamine concentrations and labeling in the temporal lobe, but not in the FCR. However, with repeated high doses (0.5 mg/kg) of MK801 no differences were found in the temporal lobe, but glutamate was increased in the FCR (Kondziella et al., 2005). In order to evaluate the significance of these findings for the understanding of glutamatergic function in schizophrenia it is important to compare with results obtained from patients. The latter analyses have to be done in vivo, where assessment of glutamatergic neurotransmission is difficult. Recent developments in NMR spectroscopy offer the opportunity to quantify a number of signals, including glutamate and glutamine. To our knowledge, there are four in vivo studies published in which absolute glutamate and glutamine concentrations were measured using NMR spectroscopy technology (Bartha et al., 1997; Theberge et al., 2002, 2003; Tebartz van et al., 2005). In an early study Bartha et al. (1997) reported increased glutamine levels in the medial prefrontal cortex of never-treated schizophrenia patients. Theberge et al. (2002) confirmed this observation for the left anterior cingulate and thalamus in a group of first-episode schizophrenia patients. In a second study, the same investigators reported significantly lower levels of glutamate and glutamine in the left anterior cingulate of patients with chronic schizophrenia, whereas Tebartz van et al. (2005) detected higher glutamate and glutamine concentrations in dorsolateral prefrontal cortex and hippocampus. However, the latter authors found an unusually low glutamate concentration in control patients and thus the relevance of this study is doubtful. Taken together the first three reports indicate that first-episode patients have an increased glutamine level in different areas of the brain, whereas chronic patients have decreased glutamate and glutamine levels in anterior cingulate. Animal models of schizophrenia should mimic this. In the present study,

increases were found in glutamate and glutamine in the temporal lobe but not in the FCR. Thus, it appears that this model does not show the same pattern as seen in schizophrenia patients and mimics more the toxic effects of MK801 possibly caused by increased glutamate in the extracellular space. In line with this is the fact that low doses of MK801 in the present study lead only to moderate hyperlocomotion alterations compared with high doses (data not shown). Repeated injection of the high dose (0.5 mg/kg) does, however, show very similar results to those from first-episode patients (Kondziella et al., 2005) and might thus be a good model for schizophrenia.

4.2. Astrocyte–neuron interactions

In the present study, labeling of glutamate and glutamine was increased in the temporal lobe, whereas GABA labeling was unchanged. It is possible to study astrocyte–neuronal interactions by analyzing the labeling patterns from [1-^{13}C]-glucose and [1,2-^{13}C]acetate in metabolites. A major role of astrocytes in the adult brain is to support neurons metabolically and the glutamate–glutamine cycle is well established (Sonnewald et al., 1997). Astrocytes are essential in maintaining the low glutamate levels in the synaptic cleft needed for precise receptor-mediated functions (Danbolt, 2001). Extracellular glutamate is taken up by astrocytes, in which it is converted to glutamine. Glutamine is then transported to the presynaptic neuron and converted to glutamate again. This process appears to be enhanced in our study, since labeling of both glutamate and glutamine from glucose, and thus mostly neurons, is increased in the temporal lobe. Labeling from [1,2-^{13}C]acetate was also increased in glutamate and glutamine in this area after repeated injection of 0.1 mg/kg MK801. Since the transport system for acetate is mostly on astrocytes such labeling originates from astrocytes (Waniewski and Martin, 1998). Taken together, these observations might be an indication of increased glutamatergic function, which could cause the neurotoxic effects of NMDA antagonism reported by several authors (Olney and Farber, 1994; Farber et al., 1995, 1996, 1998). A similar increase in labeling of glutamate and glutamine in the temporal lobe was reported by Brenner et al. (2005) after injection of a single dose of 0.5 mg/kg MK801. However, repeated high dose injections had no effect on labeling in the temporal lobe but lead to a decrease in labeling of glutamate and GABA from [1-^{13}C]glucose in the FCR (Kondziella et al., 2005).

Interestingly, localization of the changes in the metabolic results is different than that of histologic results. Several authors reported reversible vacuolization and other histologic abnormalities confined to retrosplenial cortex (RSC), which is part of the FCR, with low doses, involving the temporal lobe only when high doses were given (Olney et al., 1989; Wozniak et al., 1996, 1998). Although the mismatch between metabolic and histologically results seems surprising at first glance, it should be noted that the histological alterations were fully reversible (Wozniak et al., 1996) and that altered cell structure is not always identical with altered cell function.

4.3. Complex polysynaptic network impairments and the kindling effect

Why do different doses of MK801 lead to impairment of different brain areas? While a single high dose and repeated low doses of MK801 lead to biochemical alterations in temporal lobe, repeated high doses spared the temporal lobe largely, but evoked significant disturbances in the FCR. It is highly interesting in this context, that MK801 directly injected into the RSC, which is part of the FCR, does not produce the same neurotoxic reactions as that seen with equivalent systemic doses (Farber et al., 2002). This signifies that NMDA receptor blockade is required at one or more sites outside the RSC to trigger these neurotoxic effects. Thus, polysynaptic network impairments have to be considered. Possibly, different doses of MK801 may have the same local effects in the FCR, but different outcome in other non-FCR areas, whereby low doses of MK801 may change polysynaptic chains in such a way that metabolic disturbances are most pronounced in the temporal lobe. In contrast, high doses of MK801 affect other polysynaptic chains, leading to alterations in FCR. Since only repeated doses, but not a single dose, of 0.5 mg/kg MK801 lead to alterations in the FCR, one might postulate that the relevant polysynaptic changes have to be sensitized, or in other terms, ''kindled''.

In conclusion; do repeated MK801 injections serve as a model of schizophrenia? The answer is yes, if the chosen dose is high enough. Repeated low doses (0.1 mg/kg) of MK801 mimic the behavioral changes such as a slight hyperlocomotion and increased pre-pulse inhibition (Schulz et al., 2001), but not the neurochemical alterations seen in schizophrenia. Repeated high doses (0.5 mg/kg) mimic both. In conclusion, repeated injection of MK801 at 0.1 mg/kg appears to model the toxic effects of NMDA hypofunction, whereas repeated administration of high doses (0.5 mg/kg) mimics the results observed in first-episode schizophrenia patients.

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Paper II

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Glial–Neuronal Interactions are Impaired in the Schizophrenia Model of Repeated MK801 Exposure

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Schizophrenia-mimicking compounds such as phencyclidine (PCP) and MK801 are antagonists at the *N*-methyl-D-aspartate (NMDA) receptor and produce the whole spectrum of positive, negative, and cognitive symptoms. This is one of the most important pillars of the hypoglutamatergic hypothesis of schizophrenia. Since the synthesis of glutamate and GABA in neurons is closely connected to astrocyte metabolism, the study of astrocytic function is essential in this context. Dizocilpine-maleate (MK801) (0.5 mg/kg) was injected into rats every day for 6 days. The last dose was given together with [1-^{13}C]glucose and [1,2-^{13}C]acetate. Extracts from frontal, retrosplenial, and cingulate cortices (CRFC) and temporal lobes were examined by ^{13}C nuclear magnetic resonance spectroscopy, high pressure liquid chromatography, and light microscopy. In CRFC, significant increases in the levels of glutamate, glutathione, and taurine were seen, whereas amounts and turnover of noradrenaline, dopamine, and serotonin were unchanged. Glutamate and glutamine, derived from [1,2-^{13}C]acetate and thus astrocytes, were significantly decreased in CRFC as compared to controls. Labeling from [1-^{13}C]glucose and thus mostly neuronal metabolism was affected in the same brain region with decreased labeling of glutamate and GABA. The present model mimics the increased glutamate/glutamine activity found in drug-naive patients with first episode schizophrenia. Moreover, the decreased labeling indicates the transition to lower glutamatergic function seen in chronic schizophrenia patients. The disturbance in astrocytic function and the glutamine–glutamate–GABA cycle are of significant importance and might add to the malfunction of the cortico-striato-thalamo-cortical loop caused by NDMA receptor blockade.
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Keywords: glutamate; GABA; glutamine; dopamine; NMDA glutamate antagonists

INTRODUCTION

Evidence is accumulating that the hypothesis of exclusive dopaminergic hyperfunction causing schizophrenia has to be revised. A more differentiated approach includes, in addition, the dopaminergic, glutamatergic, GABAergic, and serotonergic neurotransmitter systems (Carlsson *et al*, 2001). Particularly, glutamatergic hypofunction has been recognized to play a significant role in schizophrenia. *N*-methyl-D-aspartate (NMDA) glutamate-receptor antagonists, such as phencyclidine (PCP), ketamine, or dizocilpine-maleate (MK801), have been shown to cause strong psychomimetic effects with hallucinations and psychomotor signs and have been used extensively in schizophrenia research. In contrast to dopaminergic agonists, which only mimic the positive symptoms of schizophrenia, NMDA antagonists produce the whole spectrum including negative and cognitive symptoms (Carlsson *et al*, 2001).

It has been suggested that the dysregulation of dopamine transmission in schizophrenia might be secondary to alterations in glutamatergic NMDA receptor-mediated transmission (Olney and Farber, 1995; Carlsson *et al*, 2004). A direct linkage between the two hypotheses has recently been shown in an experiment performed in healthy volunteers. It was observed that the amplitude of amphetamine-induced dopamine release was significantly enhanced compared to control conditions, when simultaneously the NMDA receptor-mediated transmission was decreased by ketamine administration (Kegeles *et al*, 2000). Thus, the elevated dopamine release seen in schizophrenic patients after amphetamine administration might very well be secondary to a failure in glutamatergic control of dopamine neurons. Analogous studies in rats support this interpretation (Miller and Abercrombie, 1996). NMDA receptor hypofunction models not involving dopamine have also been proposed (Carlsson and Carlsson, 1989, 1990; Olney, 1989).

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The synthesis of glutamate and GABA in neurons is closely connected to astrocyte metabolism and, thus, there are good reasons to postulate a role for astrocytes in the pathophysiology of glutamate/NMDA hypofunction and schizophrenia. Being the most important excitatory neurotransmitter in the mammalian CNS, glutamate and its homeostasis are crucial to brain function for several reasons. Firstly, fast removal of glutamate from the synaptic cleft by astrocytes guarantees short glutamate action on the postsynaptic target cell and thereby precise information signaling. Secondly, high extracellular concentration of glutamate is neurotoxic and it has been shown that excessive glutamate release plays a part in the pathophysiology of many brain disorders. Thirdly, neurons are not capable of net synthesis of glutamate and related metabolites, since they lack the main anaplerotic enzyme in the brain, pyruvate carboxylase (Shank *et al*, 1985). Thus, they depend on astrocytic supply of tricarboxylic acid (TCA) cycle intermediates since the drain of amino-acid neurotransmitters would otherwise lead to a shortage of neurotransmitter precursors (Sonnewald *et al*, 1993). Astrocytes release glutamine into the extracellular space, from where it is taken up by neurons and converted to glutamate and GABA or channeled into the TCA cycle via 2-oxoglutarate to provide carbon skeletons for the synthesis of metabolites. As mentioned earlier, after release from neurons, glutamate is cleared from the synapses by astrocytes, which transform glutamate to glutamine again, and the so-called 'glutamine–glutamate-cycle' is completed (Sonnewald *et al*, 2005).

^{13}C nuclear magnetic resonance spectroscopy (NMRS) is an excellent tool to obtain information about metabolic pathways and glial–neuronal metabolic interaction—see Sonnewald and Kondziella (2003) for a review. The natural abundance of ^{13}C is only 1.1%, thus ^{13}C-labeled precursors and products are easily detected. When [1,2-^{13}C]acetate and [1-^{13}C]glucose are given simultaneously, it is possible to study astrocytic and neuronal metabolism in the same animal. This is because acetate is exclusively taken up by astrocytes, while the major part of acetyl-CoA derived from glucose is metabolized in neurons (Taylor *et al*, 1996). Thus, ^{13}C NMRS provides a more dynamic picture of metabolic changes compared to ^{1}H NMRS and high pressure liquid chromatography (HPLC). MK801 is one of the best-characterized noncompetitive antagonists of the PCP-binding site of the NMDA receptor, which is also present on astrocytes (Krebs *et al*, 2003). Since it is even more selective than PCP (Carlsson *et al*, 2001), MK801 is a widely studied substance in experimental research of both schizophrenia and glutamate neurotoxicity. It causes behavioral changes in rodents such as hyperlocomotion, stereotyped behavior, head weaving, and ataxia (Loscher and Honack, 1991). Altered cerebral metabolism with excessive cerebral glucose supply has been reported by Loubinoux *et al* (1994). MK801 is able to minimize cell damage in the rodent hippocampus during ischemia, and also to induce neuronal degeneration. Toxic effects of MK801 were first observed and are most pronounced in the retrosplenial and cingulate cortices (Olney *et al*, 1989; Wozniak *et al*, 1998). With increasing MK801 concentrations, alterations were also seen in other brain areas including entorhinal and piriform cortices, dentate gyrus, and amygdala (Horvath *et al*, 1997).

Since the diagnosis of schizophrenia requires recurrent episodes of psychoses, a protocol with repeated injections of MK801 was chosen in the present study. We induced a state of NMDA receptor hypofunction in rats by injection of MK801 every day for 6 days. Then astrocytic–neuronal interactions in frontal, retrosplenial, and cingulate cortices and temporal lobe were examined by means of NMRS, HPLC, and light microscopy to test the hypothesis that glial–neuronal metabolism is disturbed in the schizophrenia model of repeated MK801 treatment.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats with an average weight of 250 g were obtained from Möllegaard Breeding Centre, Copenhagen, Denmark. [1-^{13}C]glucose, [1,2-^{13}C]acetate (both 99% ^{13}C enriched), and D_2O (99.9%) were purchased from Cambridge Isotopes Laboratories (Woburn, MA, USA); ethylene glycol from Merck (Darmstadt, Germany). *o*-Phthaldialdehyde and MK801 (Dizocilpine; [5*R*, 10*S*]-[+]-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine) were from Sigma-Aldrich, St Louis, MO, USA. All other chemicals were of the purest grade available from local commercial sources.

Methods

All animal procedures were approved by the Norwegian Animal Research Authority. Prior to experiments, the animals received food and water *ad libitum* and were kept at a light/dark cycle of 12 h, humidity 60%, temperature 22°C. During the experiment, the animals were housed in individual cages. Saline (10 animals) or MK801 (10 animals; 0.5 mg/kg body weight) was administered intraperitoneally (i.p.) every day for 6 days. The last dose of MK801 or saline was given in seven (MK801) and five (saline) rats together with [1-^{13}C]glucose (543 mg/kg, 0.3 M solution) and [1,2-^{13}C]acetate (504 mg/kg, 0.6 M solution) i.p. followed by decapitation 20 min later. Brains from these animals were analyzed by HPLC and ^{13}C NMRS. The heads were snap frozen in liquid nitrogen and stored at −80°C. Brains were removed, and two different areas of each hemisphere were dissected. The first area included the cingulate, the retrosplenial, and the frontal cortices (CRFC). The second area, which in the present study will be referred to as the temporal lobe, was dissected by a horizontal cut from the most lateral point of the hemisphere extending approximately 3 mm medially and a second sagittal cut extending ventrally through the whole brain. The resulting sample consisted of the temporal cortex, piriform cortex, entorhinal cortex, amygdala, and parts of the hippocampus. The dissection was performed on ice while the brains were still frozen, and lasted a maximum of 3 min. After dissection, brain tissue was homogenized in 7% (w/v) perchloric acid and centrifuged at 4000 *g* for 5 min. The procedure was repeated, the supernatants pooled and neutralized with 1 M KOH followed by lyophilization.

Histology

Three MK801-treated rats and five controls did not receive [1-^{13}C]glucose and [1,2-^{13}C]acetate in their last injection. They were killed with an overdose of equithisin and underwent cardiac perfusion with saline and 4% formaldehyde. Brains were removed immediately after perfusion and stored in the same fixative until slicing. Frozen sections were cut and stained with hematoxilin–eosin or cresyl violet. Using the atlas of Paxinos and Watson (1998) CRFC, temporal lobes and hippocampus were identified and examined by light microscopy for signs of neuronal degeneration such as intracytoplasmatic vacuoles, remnant nuclear debris, brightly eosinophilic cytoplasm, and local glial reaction, which have been described earlier (Olney, 1989).

HPLC

Total amounts of amino acids and total glutathione were determined by HPLC using the HP 1100 System from Agilent (Palo Alto, CA, USA), with fluorescence detection, after derivatization with *o*-phthaldialdehyde. Separation was performed on a ZORBAX SB-C18 (4.6×250 mm, 5 mm) column from Agilent with 50 mM sodium acetate buffer (pH 7.0) and methanol as eluents.

The monoamine transmitter substances (NA, DA, 5-HT) and acid metabolites (DOPAC, 5-HIAA, HVA) were quantified in brain tissue homogenates by HPLC separations and electrochemical detection. The analytical method is based on two chromatographic separations for amines and acids. Both systems are equipped with a reverse phase coloumn (Luna C18(2), dp 3 μm, 50×2 mm i.d., Phenomex), and electrochemical detection is accomplished at two potentials on glassy carbon electrodes (MF-1000, Bioanalytical Systems, Inc.). The aqueous mobile phase (0.4 ml/min) for the acid system contains citric acid 14 mM, sodium citrate 10 mM, MeOH 15% (v/v), and EDTA 0.1 mM. Detection potentials relative to Ag/AgCl reference are 0.45 and 0.6 V. The aqueous ion pairing mobile phase (0.5 ml/min) for the amine system contains citric acid 5 mM, sodium citrate 10 mM, MeOH 9% (v/v), decane sulfonic acid 0.45 mM, and EDTA 0.1 mM. Detection potentials relative to Ag/AgCl reference are 0.45 and 0.65 V.

^{13}C NMR Spectroscopy

Proton decoupled 150.92 MHz ^{13}C NMR spectra were obtained using a Bruker DRX-600 spectrometer after the samples had been redissolved in 200 μl D_2O (deuterated water) containing ethylene glycol 0.1% as an internal standard. Scans were accumulated with a 30° pulse angle and 30 kHz spectral width with 64 K data points. The number of scans was typically 10 000. The acquisition time was 1.08 s, the relaxation delay 0.5 s. Factors for nuclear Overhauser and relaxation effects were applied to all spectra.

Labeling Patterns

Label from [1-^{13}C]glucose can be quantified by analyzing the singlet peaks in the different resonances (Figure 1) (Sonnewald and Kondziella, 2003). In contrast, the doublets seen in the spectrum (Figure 1) are mostly derived from [1,2-^{13}C]acetate and thus astrocytic metabolism (Waniewski and Martin, 1998). Glutamine is labeled more from [1,2-^{13}C]acetate (doublet) than [1-^{13}C]glucose (singlet); the opposite is the case for glutamate and GABA. Alanine, lactate, *N*-acetylaspartate (NAA) in the C-6 position, and succinate are mainly labeled from glucose. Creatine, taurine, inositol, and the aspartate group in NAA are not labeled; the naturally abundant ^{13}C gives rise to the observed singlets (Figure 1). Since both acetyl-CoA and oxalacetate can be labeled or unlabeled, the number of possible isotopomers of the TCA cycle-derived metabolites is large and only compounds derived from the first and the second turns are presented in Figure 2. In addition to analysis of the single peaks, conclusions about the predominant metabolic pathways can be drawn from metabolic ratios (see below). [1-^{13}C]glucose is converted to pyruvate via glycolysis and can form [3-^{13}C]alanine and [3-^{13}C]lactate. Pyruvate may enter the TCA cycle via [2-^{13}C]acetyl-CoA, which will lead to the formation of [4-^{13}C]glutamate and glutamine or [2-^{13}C]GABA. After the second turn of the TCA cycle, this label will be in the [2-^{13}C] or [3-^{13}C] positions of glutamate and glutamine or in the [3-^{13}C] or [4-^{13}C] positions of GABA. Alternatively, pyruvate can be carboxylated by pyruvate carboxylase (PC) to oxalacetate, which will lead to the synthesis of [2-^{13}C]glutamate and glutamine or [4-^{13}C]GABA. [1,2-^{13}C]acetate can also be converted to acetyl-CoA, however, the product, [1,2-^{13}C]acetyl-CoA, will have two ^{13}C atoms resulting in doublet formation. Thus, [4,5-^{13}C]glutamate and glutamine or [1,2-^{13}C]GABA are formed (Figure 2). After the second turn of the TCA cycle, this label will be in the [1,2-^{13}C] or [3-^{13}C] positions of glutamate and glutamine and the [2-^{13}C] or [3-^{13}C] positions of GABA.

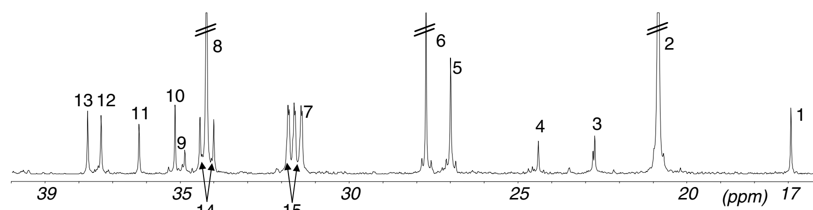

Figure 1 ^{13}C NMR spectrum of temporal lobe extracts from rats injected with MK801 together with [1,2-^{13}C]acetate and [1-^{13}C]glucose. Peak assignments: 1: alanine C-3; 2: lactate C-2; 3: *N*-acetyl-aspartate C-6; 4: GABA C-3; 5: glutamine C-3; 6: glutamate C-3; 7: glutamine C-4; 8: glutamate C-4; 9: succinate C-2/C-3; 10: GABA C-2; 11: aspartate C-3; 12: GABA C-4; 13: *N*-acetyl-aspartate C-6; 14: [4,5-^{13}C]glutamate; 15: [4,5-^{13}C]glutamine.

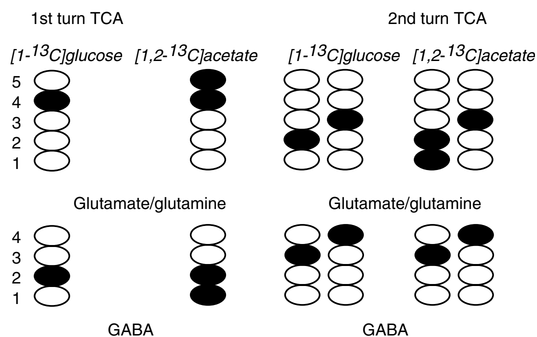

Figure 2 ^{13}C-labeling patterns in glutamate, glutamine, and GABA from [1-^{13}C]glucose or [1,2-^{13}C]acetate. Full circle represents ^{13}C and empty circle ^{12}C. First turn tricarboxylic acid (TCA) cycle: labeled acetyl-CoA condensing with unlabeled oxaloacetate; second turn: unlabeled acetyl-CoA condensing with labeled oxaloacetate.

Metabolic Ratios

The acetate *vs* glucose utilization ratio is an estimation of the relative contribution from neurons and astrocytes to glutamate, glutamine, and GABA formation (Taylor *et al*, 1996). Incorporation of [1,2-^{13}C]acetate yields [4,5-^{13}C] glutamate (peak 14 Figure 1), [4,5-^{13}C]glutamine (peak 15 Figure 1), and [1,2-^{13}C]GABA, whereas [1-^{13}C]glucose gives rise to [4-^{13}C]glutamate, [4-^{13}C]glutamine, and [2-^{13}C]GABA. The acetate *vs* glucose utilization ratios are expressed as [4,5-^{13}C]glutamate (glutamine)/[4-^{13}C]glutamate (glutamine), and [1,2-^{13}C]GABA/[2-^{13}C]GABA.

The ^{13}C cycling ratio gives an indication of how long label stays in the TCA cycle before incorporation into glutamate and glutamine. The cycling ratio for ^{13}C from [1,2-^{13}C]acetate was calculated as follows: [1,2-^{13}C]glutamate (glutamine)/[4,5-^{13}C]glutamate (glutamine). The cycling ratio for ^{13}C from [1-^{13}C]glucose was calculated as ([3-^{13}C]glutamate (glutamine)$-$[1,2-^{13}C]glutamate (glutamine))/[4-^{13}C]glutamate (glutamine). Ratios of pyruvate carboxylase and pyruvate dehydrogenase (PC/PDH ratios) give information about the importance of replacement of intermediates lost from the TCA cycle and can be calculated as ([2-^{13}C]glutamate (glutamine)$-$[3-^{13}C]glutamate (glutamine))/[4-^{13}C]glutamate (glutamine). The labeling of GABA from the second turn is identical for [1-^{13}C]glucose and [1,2-^{13}C]acetate.

Data Analysis

The amounts of ^{13}C in the different metabolites were quantified from integrals of the relevant peaks obtained from NMR spectra with ethylene glycol as an internal standard. All results are given as mean$\pm$standard deviation. Statistics were performed using the two-tailed, unpaired Student's *t*-test; $p<0.05$ was regarded as significant.

RESULTS

MK801 induced hyperlocomotion, ataxia, abducted hind limbs, flat body posture, and stereotyped behavior such as head waving, which were characterized by considerable inter- and intrasubject variability. Injection of [1-^{13}C]glucose and [1,2-^{13}C]acetate lead to efficient labeling of many metabolites (Figure 1). Particularly, labeling of glutamate and glutamine C-3 and C-4, GABA C-2 and C-3, aspartate, and lactate C-3 are shown in Figure 1. Labeling patterns from [1-^{13}C]glucose and [1,2-^{13}C]aspartate from the first and second turns of the TCA cycle are shown in Figure 2.

MK801 affected the CRFC to a much greater extent than the temporal lobe. As can be seen in Table 1, there was a significant increase in the levels of glutamate, taurine, and glutathione in the CRFC compared to control. Concentrations of serine, aspartate, alanine, NAD$^+$, ADP plus ATP (data not shown), and NAA were not affected by MK801. Levels of noradrenaline, serotonin, and dopamine remained unchanged (data not shown). This was also true for the turnover of dopamine (DOPAC/DA and HVA/DA) and serotonin (5-HIAA/5-HT). In the temporal lobe, only the amount of inositol was increased after MK801. The amounts of [4,5-^{13}C]glutamate and [4,5-^{13}C]glutamine, derived from [1,2-^{13}C]acetate, were significantly decreased in CRFC as compared to controls (Table 2). Label from [1-^{13}C]glucose was affected in the same brain region of MK801-treated rats with decreases of [4-^{13}C]glutamate, [2-^{13}C]GABA, and [4-^{13}C]GABA, whereas in the temporal lobe both [1,2-^{13}C]acetate and [1-^{13}C]glucose-derived metabolites were not altered. Concentration of [3-^{13}C]lactate was unchanged in both areas (data not shown). In Table 3, it can

Table 1 Total Amounts (μmol/g Brain Tissue) of Metabolites (a) and Turnover Rates of Dopamine and Serotonin (b) in Brain Extracts of Controls ($n=5$) and MK801-Treated Rats ($n=7$)

	CRFC		Temporal lobe	
	Control	MK801	Control	MK801
(a) *Metabolites*				
Glutamate	7.80±1.9	10.13±2.3**	8.09±1.4	9.36±1.4
GABA	1.48±0.5	1.89±0.7	2.02±0.5	2.16±0.6
Glutamine	2.59±0.9	3.22±1.1	3.03±0.9	2.90±0.4
Glutathione	1.02±0.2	1.30±0.3*	1.15±0.2	1.05±0.2
Taurine	4.35±0.8	5.20±0.9*	4.85±0.7	5.41±0.7
N-acetylaspartate	6.00±0.8	5.40±1.1	5.72±0.8	5.00±0.5
Inositol	7.01±0.7	6.99±0.6	8.26±0.8	9.30±0.6*
(b) *Turnover rates*				
DOPAC/DA	0.65±0.2	0.68±0.2	0.13±0.1	0.14±0.1
HVA/DA	1.03±0.4	0.99±0.2	0.11±0.1	0.13±0.1
5-HIAA/5-HT	0.32±0.2	0.55±0.2	0.74±0.2	0.65±0.1

Rats received MK801 (0.5 mg/kg) or saline i.p. every 24 h for 6 days. On day six [1,2-^{13}C]acetate and [1-^{13}C]glucose were administered (see Materials and Methods for details). All results were obtained by HPLC with the exception for levels of *N*-acetylaspartate and inositol (assessed by ^{13}C NMR). The results are expressed as mean±SD and were analyzed with the two-tailed unpaired Student's *t*-test. Abbreviations: CRFC = frontal/cingulate/retrosplenial cortex; DA = dopamine; DOPAC = dihydroxyphenylacetic acid; HVA = homovanillic acid; 5-HT = serotonin; 5-HIAA = 5-hydroxyindoleacetic acid; n.d. = not detected; *$p<0.05$; **$p<0.025$.

be seen that following MK801, the cycling ratio for ^{13}C from [1-^{13}C]glucose was increased for glutamate in both the investigated brain areas. Moreover, acetate/glucose ratios for glutamate and glutamine were decreased in the CRFC. However, an increase of the cycling ratio for ^{13}C from [1,2-^{13}C]acetate was found in the CRFC for glutamine. PC/PDH ratios were not changed (data not shown).

Eosin- and cresyl violet-stained histological slices from CRFC, hippocampus, and TE were examined by light microscopy, but no morphological changes were found between MK801-treated rats and controls.

DISCUSSION

In the present study repeated injections of MK801 increased amounts of glutamate in CRFC, whereas labeling of glutamate and glutamine from [1,2-^{13}C]acetate and thus astrocytic metabolism was significantly decreased in CRFC.

Table 2 Amounts (nmol/g) of ^{13}C of Tissue in Brain Extracts from Controls ($n=5$) and MK801-Treated Rats ($n=7$)

Metabolites	CRFC		Temporal lobe	
	Control	MK801	Control	MK801
[1,2-^{13}C]acetate				
[4,5-^{13}C]Glutamate	260.8±44.6	201.0±23.3***	198.0±29.4	201.5±24.3
[1,2-^{13}C]GABA	22.6±5.7	20.8±6.1	29.4±8.5	25.2±6.2
[4,5-^{13}C]Glutamine	324.0±59.5	268.4±21.1**	306.5±50.2	285.6±28.6
[1-^{13}C]glucose				
[4-^{13}C]Glutamate	800.7±137.1	666.7±68.5*	634.0±80.5	622.9±97.8
[2-^{13}C]GABA	76.2±16.9	61.1±9.3*	91.0±14.8	76.0±16.7
[4-^{13}C]Glutamine	163.8±22.2	166.0±18.4	146.8±17.5	157.3±26.6

All results were obtained by ^{13}C NMR spectroscopy. Abbreviations: see Table 1. For details, see Materials and Methods. *$p<0.05$; **$p<0.025$; ***$p<0.01$.

Labeling from [1-^{13}C]glucose and thus mostly neuronal metabolism was affected as well in the same brain region with decreased labeling of glutamate and GABA as compared to controls. A tentative conclusion, as will be discussed below, is that repeated injection of MK801 is a good animal model for first episode schizophrenics and might give insight into the transition from the acute to the chronic stage.

Glutamate–Glutamine–GABA Cycle and Glial–Neuronal Interactions

Using HPLC we found increased levels of glutamate in the CRFC. As pointed out in the Introduction, glutamate and glutamine are very closely linked metabolically. In an *in vivo* ^{1}H NMRS study it was suggested that the glutamine signal in spectra of anterior cingulate and thalamus of drug-naive first episode schizophrenia patients was increased compared to age-matched healthy controls (Theberge *et al*, 2002). However, using ^{1}H NMRS at low field strength it is not possible to fully separate glutamate and glutamine signals, whereas there is no overlap between these two amino-acid signals using high field ^{1}H NMRS or HPLC. Thus, it is possible that also in these cited studies glutamate instead of glutamine was enhanced. In contrast to drug naive patients with first episode schizophrenia, lower levels of glutamine and glutamate were found in the left anterior cingulate cortex of patients with chronic schizophrenia compared to healthy volunteers (Theberge *et al*, 2003). In view of the decrease in labeling of glutamate and glutamine observed in this study, it is tempting to speculate that decreased amounts of these metabolites would eventually result, had the rats been injected with MK801 for an even longer period. This in turn would mirror the finding of decreased glutamate and glutamine in chronic patients. Even though chronic schizophrenia in humans develops over a much longer time period, it is conceivable that the present model of repeated MK801 injection can give insight into the transition mechanisms of first episode to chronic state schizophrenia.

Table 3 Acetate/Glucose Utilization Ratios and Cycling Ratios from [1,2-^{13}C]Acetate and [1-^{13}C]Glucose from Controls ($n=5$) and MK801-Treated Rats ($n=7$)

	CRFC			Temporal lobe		
	Acetate/glucose utilization	Glucose cycling	Acetate cycling	Acetate/glucose utilization	Glucose cycling	Acetate cycling
MK801						
Glutamate	0.29±0.0**	0.53±0.0***	0.39±0.1	0.31±0.0	0.41±0.0*	0.34±0.1
Glutamine	1.62±0.1***	0.83±0.0	0.33±0.1*	2.12±0.5	0.02±0.1	0.35±0.1
GABA	0.35±0.1	0.94±0.2	ND	0.33±0.1	0.83±0.1	ND
Controls						
Glutamate	0.33±0.0	0.46±0.0	0.34±0.1	0.33±0.1	0.33±0.0	0.33±0.0
Glutamine	1.97±0.2	0.77±0.1	0.27±0.1	1.85±0.3	0.15±0.1	0.31±0.1
GABA	0.31±0.0	0.80±0.1	ND	0.33±0.1	0.85±0.1	ND

All results were obtained by ^{13}C-NMR spectroscopy. Abbreviations: see Table 1. For details see Materials and Methods. *$p<0.05$; **$p<0.025$; ***$p<0.01$.

Whereas levels of glutamate were increased in the CRFC following *repeated* MK801 administration, after a *single* dose of MK801, an increase was instead found in the temporal lobe (Brenner *et al*, 2005). In contrast to acute administration, where the amounts of labeled glutamate were not altered, repeated MK801 injections lead to decreases of both [4-^{13}C]glutamate and [4,5-^{13}C]glutamate in the CRFC. The fact that the amount of [4-^{13}C]glutamine was unchanged indicates that transfer of glutamate from neurons to astrocytes was not altered and thus an intriguing explanation might be that glutamate, released from neurons, accumulates in astrocytes. The decreased amount of [4,5-^{13}C]glutamate points towards impaired efflux of [4,5-^{13}C]glutamine from astrocytes to neurons, suggesting that the glutamine–glutamate cycle is disturbed during repeated NMDA-antagonist administration (Kosenko *et al*, 2003) and possibly schizophrenia. In line with this is the decrease of the acetate *vs* glucose utilization ratio for glutamate, which indicates lowered astrocytic contribution to glutamate formation. However, the reason for the increase in TCA cycling of precursors for glutamate from [1-^{13}C]glucose is not clear, but implies that label stays longer in the neuronal TCA cycle before incorporation into glutamate. That the glutamate-glutamine cycle is disrupted in schizophrenia patients as well is supported by the finding that activity of glutamine synthetase is decreased in brains of schizophrenic patients analyzed post mortem (Burbaeva *et al*, 2003). In this context, it must be recalled, however, that treatment with neuroleptics might contribute to a disrupted glutamate-glutamine cycle.

Glutamine released by astrocytes serves additionally as a precursor for the inhibitory neurotransmitter GABA via glutamate (Sonnewald *et al*, 1993). Therefore, the concept of the glutamate-glutamine cycle has been extended to the glutamate–glutamine–GABA cycle (Sonnewald *et al*, 2005). The amount of GABA and [1,2-^{13}C]GABA from [1,2-^{13}C]acetate remained unchanged, whereas, [2-^{13}C]GABA was decreased in the CRFC in the present study. Thus, GABA turnover from [1-^{13}C]glucose was affected, which corroborates recent reports of impaired activity of glutamic acid decarboxylase (GAD) in schizophrenic patients (Guidotti *et al*, 2000; Volk *et al*, 2000; Dracheva *et al*, 2004). In this context, it is noteworthy that a decrease in GAD_{67} has been suggested as a link between the glutamatergic and dopaminergic theories of psychosis (Hossein Fatemi *et al*, 2005).

It is interesting that the changes in the model of repeated MK801 administration did not occur in the temporal lobe as seen with acute MK801 administration (Brenner *et al*, 2005), but in the CRFC. This is in accordance with the findings in human schizophrenia patients (Stanley *et al*, 1996; Theberge *et al*, 2003; Ohrmann *et al*, 2005) and healthy subjects exposed to ketamine (Rowland *et al*, 2005). Thus, the repeated administration might generate a better schizophrenia model than acute exposure and emphasizes the importance of the frontal and retrosplenial/cingulate cortex for the pathophysiology of schizophrenia.

Dopamine and its Interaction with Glutamate Metabolism

Surprisingly, in contrast to the consequences of acute PCP exposure, repeated PCP administrations lowered dopamine turnover in the monkey and rat cortex (Jentsch *et al*, 1997a, b). NMDA antagonism appeared to be at least partly responsible for this effect, since a comparable, although smaller, reduction in dopamine turnover in the cortex of rats was seen after repeated MK801 administration (Jentsch *et al*, 1998). In these studies as well as in ours, absolute dopamine levels were unaltered. However, in the present study, metabolite/transmitter ratios (DOPAC/DA and HVA/DA) were unchanged, indicating that dopamine turnover was unaffected. Jentsch and Roth (1999) proposed that the absence of PCP-induced changes in tissue concentrations of dopamine itself implied that no direct neurotoxic insult to dopaminergic neurons occurred during the repeated PCP administration. A loss of dopaminergic neurons would otherwise be accompanied by a loss of dopamine content in the terminal fields of these projection neurons (Jentsch and Roth, 1999).

Decreased corticostriatal glutamatergic neurotransmission results in decreased negative feedback mediated via the so-called indirect striatothalamic pathway (Carlsson, 1993). Consequently, thalamic filter function is impaired. If glutamatergic NMDA receptors are dysfunctional in schizophrenia, the present and other studies suggest that secondary disturbances in glial–neuronal interactions and the glutamine–glutamate cycle may occur in this disorder (Do *et al*, 2000; Theberge *et al*, 2002, 2003). This may then contribute to imbalance of the cortico-striato-thalamo-cortical feedback as also suggested by Laruelle *et al*. (2003).

As in the study with acute exposure to MK801 (Brenner *et al*, 2005), glutathione concentration was increased during repeated MK801 injection, however, this time in the CRFC. Being the main antioxidant in the brain (Dringen and Hirrlinger, 2003), glutathione protects cells from damage by reactive oxygen species originating partly from dopamine metabolism. Decreased glutathione levels might lead to degenerative processes in the surroundings of dopaminergic terminals with loss of connectivity as a result. The increased level of glutathione in the present study could point to a decreased use of antioxidants, possibly caused by diminished NO production due to lower Ca^{2+} concentration and NMDA blockade. Interestingly, there is evidence that glutathione also enhances the NMDA receptor response to glutamate (Janaky *et al*, 1993). However, in cerebrospinal fluid of drug-free schizophrenic patients, a significant decrease in the level of glutathione was observed (Do *et al*, 2000). Combined with our results, this could indicate that transport of glutathione from the brain to CSF is disturbed.

Energy Metabolism and Histology

On histologic examinations, no signs of neuronal degeneration were found, which is in agreement with observations by other authors (Brosnan-Watters *et al*, 1999; Jentsch and Roth, 1999). Reversible changes were seen in rat brain exposed to both chronic and acute NMDA antagonist administration with neuronal vacuolar degeneration, remnant nuclear debris, and local glial reaction (Wozniak *et al*, 1996; Brosnan-Watters *et al*, 1999) and appeared at the earliest 4 h after MK801 administration (Fix *et al*, 1993). Permanent damage was only seen at doses 20 times higher than the one used in the present study (Wozniak *et al*,

1998). Since MK801 administration at low doses induces only reversible morphological changes, neuronal damage must be considered functional, not structural. Especially in light of the normal NAA concentration observed in the present study, it appears that NAA synthesis and thus neuronal mitochondrial metabolism (Baslow, 2003) is not severely affected by MK801. However, decreased labeling of glutamate, GABA, and aspartate from [1-^{13}C]glucose indicated somewhat decreased mitochondrial function in neurons in the CRFC. Glycolysis appeared normal as seen in unaltered alanine and lactate levels.

The increase of inositol in the temporal lobe is surprising since it is the only change in metabolite concentration detected in this brain region and may indicate glial swelling. Increase in inositol in the temporal lobe was also reported earlier (Brenner *et al*, 2005). Only a minor change was found in labeling in the temporal lobe in the present study, with cycling of label from [1-^{13}C]glucose being increased in glutamate.

Conclusions

Injection of MK801 and other NMDA antagonists in rodents are considered appropriate schizophrenia models because these compounds induce both positive and negative symptoms in humans in contrast to other psychomimetics such as amphetamine (Tamminga *et al*, 2003). In the present study, repeated MK801 exposure led to increased glutamate concentration in the CRFC, which mimics the results seen in first episode schizophrenic patients. The decreased levels of [4-^{13}C]glutamate, [4,5-^{13}C]glutamate, and [4,5-^{13}C]glutamine could eventually lead to the decrease in these metabolites seen in chronic patients. The present model appears very well suited to study the cascade of events taking place during the transition from first episode to chronic schizophrenia, and might help develop new treatment strategies. Furthermore, ^{13}C studies can also be carried out in humans and thus ^{13}C NMR spectroscopy might become a tool in treatment monitoring in patients. The imbalance in the glutamate homeostasis and thus impaired glial–neuronal interactions might contribute to disturbances in cortico-striato-thalamo-cortical feedback.

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156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
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160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.

163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
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168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
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183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
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187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS

193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
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198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
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204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
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212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
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214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN

218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
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223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
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225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
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227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
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232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

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235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
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237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA
244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES

245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

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252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
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255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
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257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS
260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
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267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

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269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE
271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT
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274. Birger Henning Endreseth: STRATEGIES IN RECTAL CANCER TREATMENT – FOCUS ON EARLY RECTAL CANCER AND THE INFLUENCE OF AGE ON PROGNOSIS
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276. Mansour Akbari: HUMAN BASE EXCISION REPAIR FOR PRESERVATION OF GENOMIC STABILITY
277. Stein Sundstrøm: IMPROVING TREATMENT IN PATIENTS WITH LUNG CANCER – RESULTS FROM TWO MULITCENTRE RANDOMISED STUDIES
278. Hilde Pleym: BLEEDING AFTER CORONARY ARTERY BYPASS SURGERY - STUDIES ON HEMOSTATIC MECHANISMS, PROPHYLACTIC DRUG TREATMENT AND EFFECTS OF AUTOTRANSFUSION
279. Line Merethe Oldervoll: PHYSICAL ACTIVITY AND EXERCISE INTERVENTIONS IN CANCER PATIENTS
280. Boye Welde: THE SIGNIFICANCE OF ENDURANCE TRAINING, RESISTANCE TRAINING AND MOTIVATIONAL STYLES IN ATHLETIC PERFORMANCE AMONG ELITE JUNIOR CROSS-COUNTRY SKIERS
281. Per Olav Vandvik: IRRITABLE BOWEL SYNDROME IN NORWAY, STUDIES OF PREVALENCE, DIAGNOSIS AND CHARACTERISTICS IN GENERAL PRACTICE AND IN THE POPULATION
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285. Kristin Gabestad Nørsett: GENE EXPRESSION STUDIES IN GASTROINTESTINAL PATHOPHYSIOLOGY AND NEOPLASIA
286. Per Magnus Haram: GENETIC VS. AQUIRED FITNESS: METABOLIC, VASCULAR AND CARDIOMYOCYTE ADAPTATIONS
287. Agneta Johansson: GENERAL RISK FACTORS FOR GAMBLING PROBLEMS AND THE PREVALENCE OF PATHOLOGICAL GAMBLING IN NORWAY
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289. Charlotte Björk Ingul: QUANITIFICATION OF REGIONAL MYOCARDIAL FUNCTION BY STRAIN RATE AND STRAIN FOR EVALUATION OF CORONARY ARTERY DISEASE. AUTOMATED VERSUS MANUAL ANALYSIS DURING ACUTE MYOCARDIAL INFARCTION AND DOBUTAMINE STRESS ECHOCARDIOGRAPHY
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296. Paul Jarle Mork: MUSCLE ACTIVITY IN WORK AND LEISURE AND ITS ASSOCIATION TO MUSCULOSKELETAL PAIN
297. Björn Stenström: LESSONS FROM RODENTS: I: MECHANISMS OF OBESITY SURGERY – ROLE OF STOMACH. II: CARCINOGENIC EFFECTS OF *HELICOBACTER PYLORI* AND SNUS IN THE STOMACH

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300.May Britt Drugli: YOUNG CHILDREN TREATED BECAUSE OF ODD/CD: CONDUCT PROBLEMS AND SOCIAL COMPETENCIES IN DAY-CARE AND SCHOOL SETTINGS
301.Arne Skjold: MAGNETIC RESONANCE KINETICS OF MANGANESE DIPYRIDOXYL DIPHOSPHATE (MnDPDP) IN HUMAN MYOCARDIUM. STUDIES IN HEALTHY VOLUNTEERS AND IN PATIENTS WITH RECENT MYOCARDIAL INFARCTION
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305.Elisabeth Olstad: GLUTAMATE AND GABA: MAJOR PLAYERS IN NEURONAL METABOLISM
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307.Arne Vaaler: EFFECTS OF PSYCHIATRIC INTENSIVE CARE UNIT IN AN ACUTE PSYCIATHRIC WARD
308.Mathias Toft: GENETIC STUDIES OF LRRK2 AND PINK1 IN PARKINSON'S DISEASE
309.Ingrid Løvold Mostad: IMPACT OF DIETARY FAT QUANTITY AND QUALITY IN TYPE 2 DIABETES WITH EMPHASIS ON MARINE N-3 FATTY ACIDS
310.Torill Eidhammer Sjøbakk: MR DETERMINED BRAIN METABOLIC PATTERN IN PATIENTS WITH BRAIN METASTASES AND ADOLESCENTS WITH LOW BIRTH WEIGHT
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312.Olav Magnus Søndenå Fredheim: HEALTH RELATED QUALITY OF LIFE ASSESSMENT AND ASPECTS OF THE CLINICAL PHARMACOLOGY OF METHADONE IN PATIENTS WITH CHRONIC NON-MALIGNANT PAIN
313.Anne Brantberg: FETAL AND PERINATAL IMPLICATIONS OF ANOMALIES IN THE GASTROINTESTINAL TRACT AND THE ABDOMINAL WALL
314.Erik Solligård: GUT LUMINAL MICRODIALYSIS
315.Elin Tollefsen: RESPIRATORY SYMPTOMS IN A COMPREHENSIVE POPULATION BASED STUDY AMONG ADOLESCENTS 13-19 YEARS. YOUNG-HUNT 1995-97 AND 2000-01; THE NORD-TRØNDELAG HEALTH STUDIES (HUNT)
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318. Torbjørn Dahl: CAROTID ARTERY STENOSIS. DIAGNOSTIC AND THERAPEUTIC ASPECTS
319.Inge-Andre Rasmussen jr.: FUNCTIONAL AND DIFFUSION TENSOR MAGNETIC RESONANCE IMAGING IN NEUROSURGICAL PATIENTS
320.Grete Helen Bratberg: PUBERTAL TIMING – ANTECEDENT TO RISK OR RESILIENCE ? EPIDEMIOLOGICAL STUDIES ON GROWTH, MATURATION AND HEALTH RISK BEHAVIOURS; THE YOUNG HUNT STUDY, NORD-TRØNDELAG, NORWAY
321.Sveinung Sørhaug: THE PULMONARY NEUROENDOCRINE SYSTEM. PHYSIOLOGICAL, PATHOLOGICAL AND TUMOURIGENIC ASPECTS
322.Olav Sande Eftedal: ULTRASONIC DETECTION OF DECOMPRESSION INDUCED VASCULAR MICROBUBBLES
323.Rune Bang Leistad: PAIN, AUTONOMIC ACTIVATION AND MUSCULAR ACTIVITY RELATED TO EXPERIMENTALLY-INDUCED COGNITIVE STRESS IN HEADACHE PATIENTS
324.Svein Brekke: TECHNIQUES FOR ENHANCEMENT OF TEMPORAL RESOLUTION IN THREE-DIMENSIONAL ECHOCARDIOGRAPHY
325. Kristian Bernhard Nilsen: AUTONOMIC ACTIVATION AND MUSCLE ACTIVITY IN RELATION TO MUSCULOSKELETAL PAIN

326. Anne Irene Hagen: HEREDITARY BREAST CANCER IN NORWAY. DETECTION AND PROGNOSIS OF BREAST CANCER IN FAMILIES WITH *BRCA1*GENE MUTATION
327. Ingebjørg S. Juel : INTESTINAL INJURY AND RECOVERY AFTER ISCHEMIA. AN EXPERIMENTAL STUDY ON RESTITUTION OF THE SURFACE EPITHELIUM, INTESTINAL PERMEABILITY, AND RELEASE OF BIOMARKERS FROM THE MUCOSA
328. Runa Heimstad: POST-TERM PREGNANCY
329. Jan Egil Afset: ROLE OF ENTEROPATHOGENIC *ESCHERICHIA COLI* IN CHILDHOOD DIARRHOEA IN NORWAY
330. Bent Håvard Hellum: *IN VITRO* INTERACTIONS BETWEEN MEDICINAL DRUGS AND HERBS ON CYTOCHROME P-450 METABOLISM AND P-GLYCOPROTEIN TRANSPORT
331. Morten André Høydal: CARDIAC DYSFUNCTION AND MAXIMAL OXYGEN UPTAKE MYOCARDIAL ADAPTATION TO ENDURANCE TRAINING

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